METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN AND AZITHROMYCIN BY RP-HPLC IN TABLET DOSAGE FORM

A Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

CHENNAI- 600 032.

In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch V – PHARMACEUTICAL ANALYSIS

Submitted by

Name : S. RADHAKRISHNAN

Reg. No. 261630203

Under the guidance of

Dr. CAROLINE NIMILA, M.Pharm., Ph.D.,

Associate Professor

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

J.K.K. NATTRAJA COLLEGE OF PHARMACY,

KOMARAPALAYAM – 638 183.

TAMILNADU.

MAY - 2018.

CONTENTS

LIST OF TABLES

ABBREVIATIONS

RSD	:	Relative standard deviation
HPLC	:	High performance liquid chromatography
ICH	:	International conference on harmonization of technical requirements for registration of pharmaceuticals for human use.
Q2R1		Validation of analytical procedures: Text and methodology
USP	:	United states pharmacopeia
IHS	:	In House Specification
R & D	:	Research and Development
N	:	Normality
M	:	Molarity
WS/RS	:	Working standard/Reference standard
SPL	:	Sample
STD	:	Standard
ASB	:	As such basis
AR	:	Analytical grade
STP	:	Standard testing procedure
Wt	:	Weight
nm	:	Nanometer
Mg	:	Milligram
cm	:	Centimeter
Std dev	:	Standard Deviation
µL	:	Micro liter

1. INTRODUCTION

1. 1 INTRODUCTION

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The current good manufacturing practice (CGMP) and Food Drug Administration (FDA) Guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Development of a method of analysis is usually based on prior art (or) existing literature, using the same (or) quite similar instrumentation .It is rare today that an HPLC-based method is developed that does not in same way relate (or) compare to existing, literature based approaches. Today HPLC (High performance liquid chromatography) is the method of choice used by the pharmaceutical industry to assay the intact drug and degradation products. The appropriate selection and chromatographic conditions ensure that the HPLC method will have the desired specificity. UV spectroscopy is also a simple analytical tool widely used for routine assay of drugs. Hence for the assay of the selected drugs HPLC and UV spectroscopy has been chosen for these proposed methods.

The developed chromatographic methods further validated as per ICH or USFDA guidelines for all the critical parameters. To access the precision and to evaluate the results of analysis the analyst must use statistical methods. These methods include confidence limit, regression analysis to establish calibration curves. In each analysis the critical response parameters must be optimized and recognized if possible.

Pharmaceutical Analysis plays a major role today, and it can be considered as an interdisciplinary subject. Pharmaceutical analysis derives its principles from various branches like chemistry, physics. And microbiology etc. pharmaceutical Analytical techniques are applied mainly in two areas, quantitative analysis and qualitative analysis, although there are several other applications.

Drugs and pharmaceuticals are chemicals or like substances, which or of organic inorganic or other origin. Whatever may be the origin, we some property of the medicinal agent to measure them quantitatively or qualitatively.

In recent years, several analytical techniques have been evolved that combined two or more methods into one called "hyphenated" technique eg: GC/MS, LC/MS etc. The complete Analysis of a substance consists of four main steps.

The concept of analytical chemistry lies in the simple, precise and accurate measurements. These determinations require highly sophisticated instruments and methods like mass spectroscopy, gas chromatography, HPTLC, HPLC, etc. HPLC method is sensitive, accurate, precise and desirable for routine estimation of drugs in formulations.

Thereby it is advantageous than volumetric methods. Many HPLC methods has been developed and validated for the quantitative determination of various marketed drugs.

Analytical method development and validation places an important role in drug discovery and manufacture of pharmaceuticals. These methods are used to ensure the identity, purity, potency and performance of drug products majority of analytical development effort goes into validating a stability indicating method. So it

is a quantitative analytical method based on the structure and chemical properties of each active ingredient of the drug formulation.

Most of the drugs can be analyzed by HPLC method because of several advantages like rapidity, specificity, accuracy, precision, reproducibility, ease of automation and eliminates tedious extraction and isolation procedures.

On the literature survey, it was found that most of the analytical method available for the above mentioned drug is applicable for quantification in plasma samples, the most widely used method being liquid chromatography-mass chromatography. So it is felt that there is a need to develop accurate, precise analytical methods for the estimation of the drug in solid dosage formulation.

Newer analytical methods are developed for these drugs or drug combinations of the below reasons:

- There may not be suitable method for a particular analyte in the specific matrix.
- Existing method may be too error prone or unreliable (have poor accuracy and precision).
- Existing method may be expensive, time consuming, energy intensive and may not be provide sensitive or analyte selectivity, and not easy for automation.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods.
- There may be need for an alternate method to confirm, for legal and scientific reasons.

The newly developed analytical methods having their importance in different fields that include, research and development centre (R&D), Quality control department (QC). Approved testing laboratories, chemical Analysis laboratories etc. For analysis of these drugs different analytical methods are routinely being used.

The analytical methods are classified as classical and instrumental. These methods signal measured in those methods was mentioned in following table.

Table 1: Classification of analytical method

Measurement signal	**Analytical method**
Chromatographic techniques	
Electrical	Gas chromatography (Thermal conductivity detector)
Increase in electrical current	Gas chromatography (Flame ionization detector)
Decrease in electrical current	Gas chromatography (Flame capture detector)
Electromagnetic radiation absorbed	Liquid chromatography (Ultraviolet Light detector, Diode array detector)
Electrical	Ion chromatography
Spectrophotometric method	
Emission radiation	Emission spectroscopy (X-ray, UV, Visible), Fluorescence and phosphorescence (X-ray, UV, Visible), radiochemistry.
Absorption of radiation	Spectrophotometry (X-ray, UV, Visible, IR)NMR and Electron spin resonance spectroscopy.
Scattering of radiation	Turbidimetry, Nephelometry, Raman spectroscopy
Refraction of radiation	Refractometry, Interferometry
Diffraction of Light	X-ray and Electron diffraction
Rotation of radiation	Polarimetry, Optical rotatory dispersion
Mass to charge ratio	Mass spectroscopy
Electro chemical techniques	
Electrical potential	Potentiometry
Electrical current	Polarography, Amperometry
Electrical resistance	Conductometry
Miscellaneous techniques	
Rate of reaction	Kinetic method
Thermal properties	DTA and DSC
Classical methods	
Mass	Gravimetric Analysis
Volume	Volumetric Analysis

1.2 CHROMATOGRAPHY

Techniques related to chromatography have been used for centuries to separate materials such as dyes extracted from plants. Russian botanist Tswett is credited with the discovery of chromatography. In 1903 he succeeded in separating leaf pigments using a solid polar stationary phase, It was not until 1930s that this technique was followed by Kuhn and Leder as well as Reichstein and van Euw for the separation of natural products. Martin and synge were awarded the Nobile prize for their work in 1941 in which they described liquid-liquid chromatography. Martin and synge applied the concept of theoretical plates as a measure of chromatographic efficiency. The term "chromatography" (Color-Writing derived from the Greek for Color-chroma and Write-Graphing).

CHROMATOGRAPHY IN THE PHARMACEUTICAL WORLD

In the modern pharmaceutical industry, chromatography is the major and integral analytical tool applied in all stages of drug discovery, development, and production. The development of new chemical entities (NCEs) is comprised of two major activities. Drug discovery and development. The goal of the drug discovered is to investigate a plethora of compounds employing fast screening approaches, leading to generation of lead compounds and then narrowing the selection through targeted synthesis and selective screening (lead optimization). The main functions of drug development are to completely characterize candidate compounds by performing drug metabolism, preclinical and clinical screening, and clinical trails. Throughout this drug discovery and development paradigm, rugged analytical HPLC separation methods are developed, at each phase of development to analyses of a myriad of samples are performed to adequately control and monitor the quality

of the prospective drug candidates, excipients, and final products. Effective and fast method development is of paramount importance throughout. This drug development life cycle. This requires a thorough understanding of HPLC principles and theory which have solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization.

1.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1.3.1Brief Historical prospective of chromatography:

The historical development of liquid chromatography has been extensively reviewed and can be traced as far back as they early 1900, where the Russian botanist Zwett used a variant of liquid chromatography to separate some colored plant substances.

The focus was on modern development in HPLC, a term that was coined in late 1960s with the advent of more sophisticated instrumentation, better engineered separation columns, and reliable and highly efficient stationary phases and packaging materials.

These technological advances have been, In part, fuelled, by the need to separate an increasingly large variety of differing compounds classes encountered as API s, e.g. Antibiotic, sulphonamides nucleosides, fat soluble vitamins neutral and non polar compounds. Additional challenges include developing faster and more consistent HPLC methods requiring higher flow rates, while maintaining peak shape, peak symmetry and efficiencies. Another important analytical challenge is the desire to detect and accurately quantify low levels of impurities at level present in API materials.

High-pressure liquid chromatography quickly improved with the development of column packing materials. Additional convenience of on-line detectors became rapidly a powerful separation technique and is today called as High-performance liquid chromatography (HPLC)

- One of the early problems with liquid state chromatography was the slow rate at which analysis took place. Early methods use gravity feed, and it was not uncommon diffusion and soon.

- This problem was largely overcome by the advent High-performance liquid Chromatography (HPLC). In this system the pressure is applied to the column forcing the mobile phase through at much higher rate.

- For an analysis to take several days to complete. This led not only to great delay but also the excessive time on the column and thus inevitably led to loss of resolution by

Table: 2. Different types of chromatographic techniques

S.NO	Basic principle involved	Type of chromatography
1	Techniques by Chromatographic bed shape	Column chromatography
		Paper chromatography
		Thin layer chromatography
2	Techniques by physical state of mobile phase	Gas chromatography
		Liquid chromatography
3	Affinity chromatography	Super critical fluid chromatography
4	Techniques by separation mechanism	Ion Exchange chromatography
		Size Exclusion chromatography
5	Special techniques	Reversed phase chromatography
		Two-dimensional chromatography

HPLC

In High-performance liquid chromatography, mobile as well as the stationary phase compete for the distribution of the sample components. In case of HPLC, separation is based on adsorption and partition. Adsorption chromatography employs high-surface area particles that adsorb the solute molecules. Usually a polar solid such as silica gel, alumina or porous glass beads and a non-polar mobile phase such as heptanes, octane or chloroform are used in adsorption chromatography.

In partition chromatography, the solid support is coated with a liquid stationary phase. The relative distribution of solutes between the two liquid phases determines the separation. The stationary phase can either polar or non-polar. If the stationary phase is non-polar, it is called normal phase partition chromatography. If the opposite case holds, it is called reversed-phase partition chromatography. In normal phase mode, the polar molecule partition preferentially in to the stationary phase and are retained longer than non-polar compounds. In reverse phase partition chromatography, the opposite behavior is observed.

1.3.2TYPES OF HPLC TECHNIQUES:

Based on modes of chromatography:

- Normal phase chromatography
- Reverse phase chromatography

Based on principle of separation:

- Adsorption chromatography
- Ion exchange chromatography

- Size exclusion chromatography
- Affinity chromatography

Based on elution technique:

- Isocratic separation
- Gradient separation

Based on the scale of operation:

- Analytical HPLC
- Preparative HPLC

Ion Exchange chromatography: Due to differences in the affinity of ions for the in exchange.

Size Exclusion chromatography: Due to differences in molecular weight and size of the molecules to be separated.

Affinity chromatography: Separation is based on a chemical interaction specific to the target species. The more popular revered phase mode uses a buffer and an added counter-ion of opposite charge to the sample with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvents(s).

Chairal chromatography: Separation of the enantiomers can be achieved on chairal stationary phases by the formation of diastereomers.

Analytical HPLC: only analysis of the samples is done. Recovery of the samples for reusing is normally not done.

1.3.3 MOST COMMONLY USED METHODS IN HPLC

Normal phase chromatography:

For a polar stationary bed like silica we need to choose a relatively non-polar Mobile phase. This mode of operation is termed as Normal phase chromatography. Here the least polar component elutes first, and increasing the mobile phase polarity leads to decrease in elution time. Non-polar solvents like pentane, Hexane, isooctane, cyclohexane, etc. are more popular. It is mainly used for separation of nonionic, non-polar to medium polar substances.

Reverse phase chromatography:

In 1960s, chromatographers started modifying the polar nature of the silanol group by chemically reacting silicon with organic silanes. The object was to make silica less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the reverted, the chromatographic separation carried out with such silica is referred to as Reverse-phase chromatography. Here the most post components elutes first. Increasing mobile phase polarity leads to decrease In elution time. Common solvents used in this mode include Methanol /Acetonitrile /Isopropanol etc. Mostly used for separation of ionic and polar substances. The parameters that govern the retention in reversed phase system are the following:

a. The chemical nature of the stationary phase surface.

b. The type of solvents that compose the mobile phase.

c. pH and ionic strength of the mobile phase.

Isocratic elution: A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition).

Gradient elution: The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution.

1.3.4 **INSTRUMENTATION OF HPLC:**

The mobile phase components HPLC instrument and their working functions are described below.

- Mobile phase and reservoir
- Solvent degassing system
- Pump
- Injector
- Colum
- Detector
- Data system

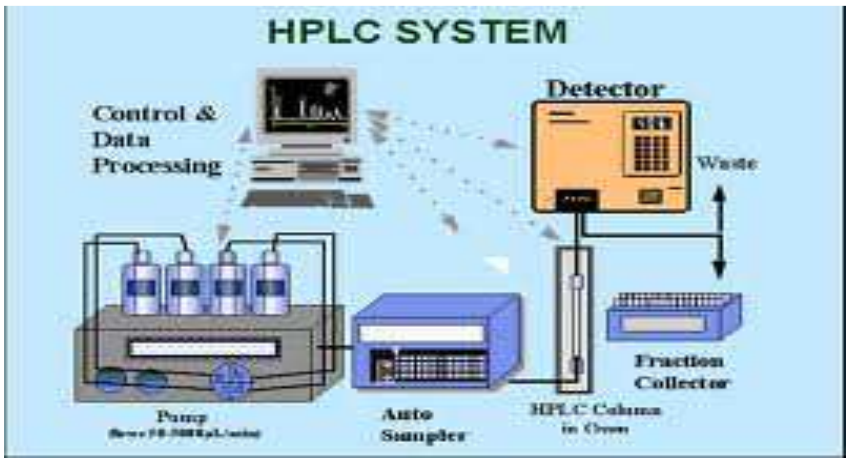


Figure1: Schematic diagram of HPLC instrumentation

I.MOBILE PHASE AND RESERVIOR:

The most common type of solvent reservoir is a glass bottle. The mobile phase is pumped under pressure from one of several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure drop across a chromatography column. Mobile phase used for HPLC are typically mixtures of organic solvents and water or aqueous buffers. The following points should also be considered when choosing a mobile phase:

- The essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.
- Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation, which can damage HPLC equipment.
- The mobile phase should have a pH 2.5 and Ph 7.0 to maximize the lifetime of the column.
- Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile when possible minimizes the absorbance of buffer.
- Use volatile mobile phase when possible, to facilitate collection of products and LC-MS analysis. Volatile mobile phases include ammonium acetate, ammonium phosphate, formic acid, and trifluoroacetic acid. Some caution is needed as these buffers absorb below 220nm.

Mobile phase used for HPLC are typically mixtures of organic solvents and water or aqueous Buffers. Physical properties of some HPLC solvents were summarized in

Table: 3.Physical properties of common HPLC solvents

Solvent	MW	BP	RI ($25^{O}C$)	UV λ Cut-off(nm)	Density g/ml($25^{O}c$)	Viscosity C_P($25^{O}C$)	Dielectric Constant
Acetonitrile	41.0	82	1.342	190	0.787	0.358	38.8
Dioxane	88.1	101	1.420	215	1.034	1.26	2.21
Ethanol	46.1	78	1.359	205	0.789	1.19	24.5
Ethyl Acetate	88.1	77	1.372	256	0.901	0.450	6.02
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
CH_2CI_2	84.9	40	1.424	233	1.326	0.44	8.93
Isopropanol	60.1	82	1.375	205	0.785	2.39	19.9
n-propanol	60.1	97	1.383	205	0.804	2.20	20.3
THF	72.1	66	1.404	210	0.889	0.51	7.58
a :The wavelength at which the absorbance of 1cm is 1.0							

II. SOLVENT DEGASSING SYSTEM:

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45µfilters, vacuum degassing with an air-soluble membrane, helium purging ultra signification or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

III. PUMP:

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.

The degree of flow of control also varies with pump expense. More expensive pumps include such state of the art technology as electronic feedback and multithreaded configurations. It is desirable to have an integrated degassing system, either helium purging, or membrane filtering.

IV. INJECTOR:

Sample introduction can be accomplished in various ways. The simplest method. Is touse an injection value in more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors in liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. Sample introduction techniques can be used with a syringe an injection valve.

V. COLUMN:

The heart of the system in the column. Many different reverse phase columns will provide excellent specificity for any particular separation. It is therefore best to routinely attempt separations with a standard C8 or C18 column and determine if it provides good separations. Reverse phase columns differ by the carbon chain length, degree of end capping and percent carbon loading. Diol, cyano and amino groups

can also be used for reverse phase chromatography. Typical HPLC columns are 5, 10, 15, and 25cm in length and are filled with small diameter (3, 5 or 10µm) particles. The internal diameter of the columns is usually 4.6mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed.

VI. DETECTOR:

The detection of UV light absorbance offers both convenience and sensitivity for molecules. When a chromophore is present, the wavelength of detection for a drug should be based on its UV spectrum in the mobile phase and not in pure solvents. The most selective wavelength for detecting a drug is frequently the longest wavelength maximum to avoid interference from solvents, Buffers and Excipient. Other method of detection can be useful are required in some instances.

1. Solute specific detectors (UV-Vis, Fluorescence, Electrochemical, Infra-red, Radio activity)
2. Bulk property detectors (Refractive index, Viscometer, conductivity)
3. Desolvation detector (Flame ionization etc)
4. LC-MS detectors
5. Reaction detectors

VII. DATA SYSTEM:

Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a

form for highly sophisticated computer analysis at a later time. The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention.

PERFORMANCE CALCULATIONS:

Calculating the following values (which can be included in a custom report) used to access overall system performance.

1. Relative retention
2. Theoretical plates
3. Capacity factor
4. Resolution
5. Peak asymmetry
6. Plates per meter

The following information furnishes the parameters used to calculate these system performance values for the separation of two chromatographic components. (Note: where the terms w and t both appear in the same equation they must be expressed the same units)

System suitability parameters:

The theory of chromatography has been used as the basis for system-suitability tests, which are set of quantitative criteria that test the suitability of the chromatographic system to identify and quantify drug related samples by HPLC at any step of the pharmaceutical analysis.

1. Relative retention: The time elapsed between the injection of the sample components in to the column and their detection is known as the retention time (Rt).

$$\alpha = (t_2-t_a) / (t_1-t_a)$$

Where,

α =Relative retention

t_1 = Retention time of the one peak measured from point of injection.

t_2 = Retention time of the second peak measured from point of injection.

t_a = Retention time of an inert peak not retained by the column, measured from point of injection.

2. Theoretical plates:

$$n = 16 (t_R / w)^2$$

Where,

n =Theoretical plates

t_R = Retention time of the component

W = width of the base of the component peak using tangent method.

3. Capacity factor: The capacity factor describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column.

$$K^1 = (t_2/t_a)-1$$

Where,

K^1 = Capacity factor

t_a = Retention time of an inert peak not retained by the column, measured from point of injection.

4. Resolution: the gap between two peaks

$$R = 2 (t_2 - t_1) / (w_2 - w_1)$$

Where,

R =Resolution between a peak of interest (peak 2) and the peak preceding it (peak 1)

W_2 = Width of the base of component peak 2

W_1 = Width of the base of component peak 1

5. Peak asymmetry

$$T = W_{0.05} / 2f$$

Where,

T = Peak asymmetry, or tailing factor.

$W_{0.05}$ = Distance from the leading edge to the tailing edge of the peak, measured at a point 5 % of the peak height from the baseline.

f= Distance from the peak maximum to the leading edge of the peak.

6. PLATE PER METER:

$$N = n/L$$

Where,

N = plates per meter.

L = column length in meters.

Advantages:

- HPLC separations can be accomplished in a minutes, in some cases even in seconds.
- High resolution of complex sample mixture into individual components.
- Rapid growth of HPLC is also because of its ability to analyse substances that are unsuitable for Gas Liquid chromatographic (GLC) analysis due to non-volatility or thermal-instability.
- Quantitative analyses are easily and accurately performed and errors of less than 1 % are common to most HPLC methods.
- Depending on sample type and detector used, it is frequently possible to measure 10^{-9} g or 1 ng of sample. With special detectors, analysis down to 10-12 pg has been reported.
- As HPLC is versatile, it can be applied to wide variety of samples like organic, inorganic, high molecular weight liquids, solids and ionic-nonionic compounds.

Disadvantages:

- HPLC instrumentation is expensive and represents a major investment for many laboratories.
- HPLC cannot handle gas samples.
- HPLC is poor identifier. It provides superior resolution but it does not provide the information that identifies each peak.
- Only one sample can be analysed at a time.

Finally, at present there is no universal and sensitive detector.

1.4 ANALYTICAL METHOD DEVELOPMENT

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trail runs are conducted, method is optimized and validated.

When alternate method proposed is intended to replace the existing procedure, comparative laboratory data includes merits /demerits should be made available.The important factors, which to be taken into account to obtain reliable quantitative analysis, are

1. Careful sample and sample preparation
2. Appropriate choice of the column
3. Selection flow rate
4. Selection of detector wavelength
5. Selection of column temperature

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. Analyte standard characterization.

a) All known information about the analyte and its structure is collected i.e., physical and chemical properties.

b) The literature for all type of information related to the analyte is surveyed.

c) Using the information in the literatures and prints, methodology is adapted. The methods are modified where ever necessary.

d) The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory SOP's are verified.

HPLC method development is based on few basic steps which include:

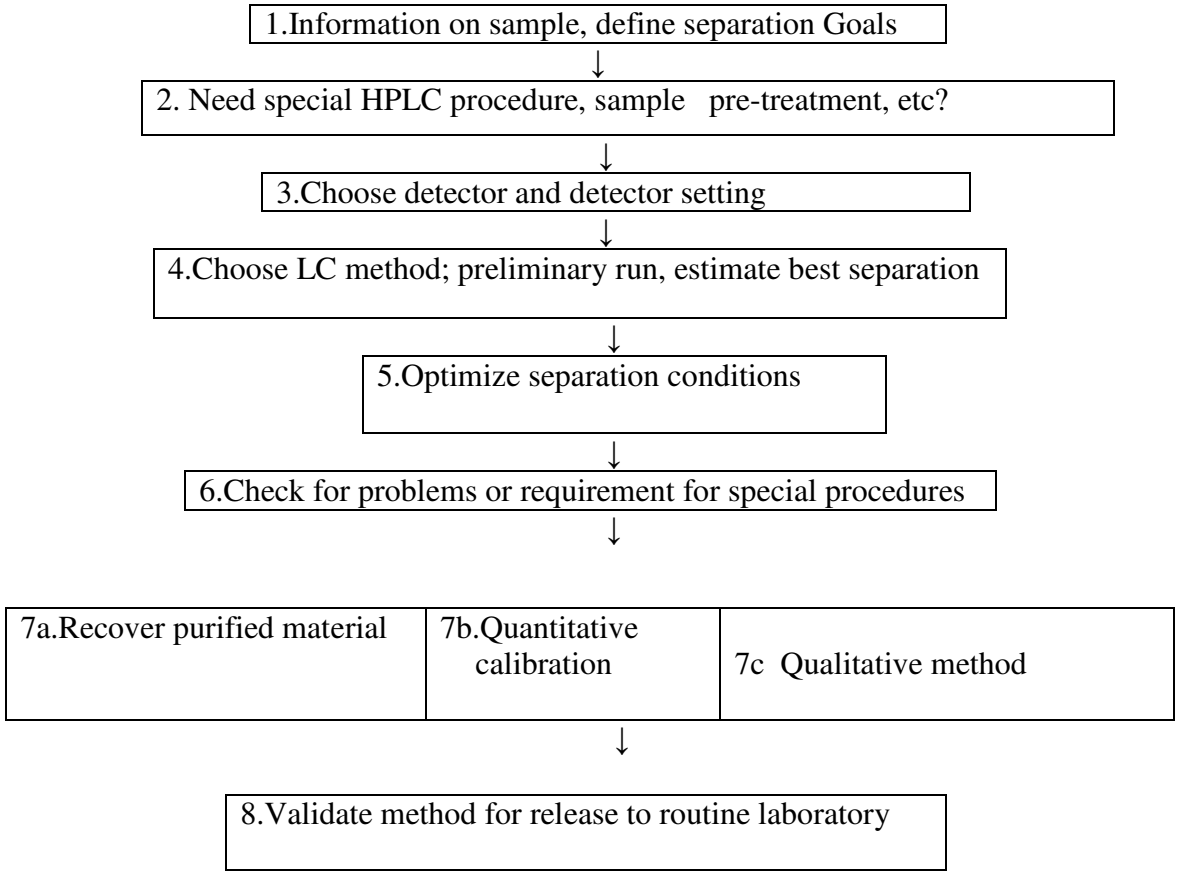


Figure2. Steps in HPLC method development

Method goals:

Analytical method goals are often defined as method acceptance criteria for peak resolution, precision, specificity, sensitivity. For instance, pharmaceutical methods for potency assays of an API typically require the following:

- Minimal sample work-up (extra and inject if possible
- Robust method that doesn't require extensive execution.
- Low cost per analysis.

Table 4: Separation goals in HPLC method development

Goals	**Comment**
Resolution	Precise and rugged quantitative analysis requires that Rs be greater than 1.5.
%RSD	Precision of retention time and peak area, <1-2%RSD.
Range	Linearity in the range of 50-150% of the lab label claim.
Analysis time	□5-30min (□60min for complex samples)
Separation time	<5-10min is desirable for routine procedures.
Quantification	≤2 %(%RSD) for assays, ≤5% for less demanding analyses, ≤15% for trace analyses.
Pressure	<150 bar is desirable, <200bar is usually essential(new column assumed)
Peak height	Narrow peaks are desirable for large signal/ noise ratios

Sample analyte information:

The information is useful for the selection of appropriate sample preparation procedures as well as the initial detection and chromatographic modes. If data not available (e.g., Pka solubility), separate studies should be initiated as soon as possible. The sample related information is summarized in Table4.

Table 5: Sample and analyte information

Sample/analyte	**Information**
Sample	Number of components concentration range of analytes
Analyte (s)	Chemical structure, molecular weight and functional groups Pka Solubility Chromophore, wavelength (max) Chairal centers, isomers Spectral data (MS,NMR, IR, and UV) Stability and toxicity
Others	Purity of reference standard materials

1. Careful sampling and sample preparation:

Before beginning method development, it is need review what is known about the sample in to define the goals of separation. The sample related information that is important to summarized in

Table: The chemical composition of the sample can be providing valuable clues for the best choice of initial conditions for an HPLC separation.

- Number of compounds present
- Molecular weight of compounds
- Pka values of compounds
- UV spectra of compounds
- Concentration range of compounds in samples of interest

2. Separation goals

The goals of HPLC separation need to be specified clearly, which include

- The use of HPLC to isolate purified sample components for spectral identification Or quantitative analysis
- It may necessary to separate all degradants or impurities from a product for reliable content assay or not
- In quantitative analysis, the required levels of accuracy and precision should be known
- Whether a single HPLC procedure is sufficient for raw materials or one or more different procedures are desired for formulations
- When the number of samples for analysis at one time is greater than 10, a run time of less than 20 minutes often will be important. Knowledge on the desired HPLC equipment.
- HPLC equipment, HPLC experience and academic training do to operators have

3. Sample preparation: samples come in various forms

- Solutions ready for injection
- solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation
- solids must be dissolved or extracted
- Samples that require pretreatment to remove interference and / or to protect the column or equipment from damage

4. Appropriate choice of the column:

The selection of the column in HPLC is somewhat similar to the selection of column in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interaction and hydrogen bond information.

Column plays the important role in achieving the chromatographic separation.

The following parameters should be considered while selecting a column:

i. length and diameter of the column

ii. packing material

iii. size and shape of the particles

iv. pore size, surface area and end capping

v. percentage of carbon loading

Columns with silica as a packing material used widely in normal phase chromatography, where the eluent (mobile phase) is non-polar consisting of various organic solvents and the stationary phase is polar. The silanol groups on the surface of the silica give it a polar character.

In reverse phase chromatography a wide variety of columns is available covering a wide range of polarity by cross linking the silanol groups with alkyl chains like $_{C6,\ C8,\ C18}$ and Nitrile groups (-CN), phenyl groups ($-_{C6H6}$) and amino groups ($-NH_2$)

ORDER OF THE SILICA BASED COLUMNS

I------Non polar------Moderately polar------Polar------I

C_{18}<C_8<C_6< Phenyl < Amino < Cyano <Silica

5. Selection of flow rate.

Flow rate is selected based on the follows:

- Retention time
- Column composition
- Separation impurities
- Peak symmetry

Preferably flow rate shall not be more than 2.5 ml/min. a flow rate that gives least retention times, good peak symmetries, least back pressure and better separation of impurities from API peak shell be selected.

6. Selection of detector wavelength:

Selection of detector wavelength is a critical step in finalization of the analytical method. To determine the exact wavelength standard API is injected into chromatographic system with photo Diode array detector and the wavelength, which gives higher response for the Compound

7. Selection of column temperature:

Ambient temperature is always preferred as a column temperature. However if the peak Symmetry could not be achieved then the column temperature can be

varied between 30^0To $_{80}0$ c. if a column temperature above $_{80}0$c is found necessary, packing material which can Withstand to that temperature shall be chosen. The increase in column temperature generally will result in reduction in peak asymmetry and peak retentions.

1.5ANALYTICAL METHOD VALIDATION:

Method validation can be defined as (ICH) "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting is predetermined specifications and quality characteristics".

Method validation study include system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

VALIDATION DEFINITION:

FDA defines validation as "Establishing documented evidence, which Provides a high degree of degree of assurance that a specific process will consistently produce a product of predetermined specifications and quality attributes.

OBJECTIVE OF METHOD VALIDATION:

The objective of validation is to form a basis for written procedure for production and control, which are designed to assure that the drug products have the identity, Quality, and purity.

TYPES OF ANALYTICAL PROCEDURES:

i. Identification tests

ii. Quantitative tests for impurities content

iii. Limit test for control of impurities

iv. Quantitative tests of the active moiety in samples of drug substances or drug product or

v. Other selected components(s) in the drug product.

vi. Dissolution testing for drug products

vii. Particle size determination for drug substances.

6. VALIDATION PARAMETERS (ICH).

Typical validation study include system suitability

I. Accuracy

II. Precision

III. Specificity

IV. Linearity

V. Detection limit

VI. Quantitation limit

VII. Range

VIII. Robustnes

1. System suitability

Prior to the analysis of samples of each day, the operator that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation has been completed.

Table: 6. System suitability parameters and Recommendation

Parameter	**Recommendation**
Capacity factor	The peak should be well-resolved from Other peaks and the void volume, Generally K>2.0
Repeatability	RSD ≤1% N ≥ 5 is desirable
Relative retention	Not essential as long as the resolution is Stated
Resolution	RS of >2 between the peak interest and The closes to eluting potential Interference (impurity, excipient, degradation product, internal standard, etc,)
Tailing factor	T of ≤ 2
Theoretical plates	N> 2000

Non-Interference of placebo:

The portion of specificity evaluation applies to the finished drug product only.Excipients present in the formulation should be evaluated and must not interfere with the detection of the analyte.

2. Linearity

The linearity of a method is a measure of how well a calibration plot of response VS concentration approximates a straight line. Linearity can be assessed by performing Single measurement at several analyte concentrations. The data is then processed using a linear least- squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

3. Precision

Precision can be defined as "The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample". A More comprehensive definition proposed by the international conference on Harmonization (ICH) divides precision into three types.

1. Repeatability

2. Intermediate precision and

3. Reproducibility

Repeatability: is the precision of a method under the same operating conditions over a short period of time.

Intermediate precision: is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory.

Reproducibility: examine the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

4. Accuracy:

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies.

There are three ways to determine accuracy:

1. Comparison to a reference standard

2. Recovery of the analyte spiked into black matrix or

3. Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined. e.g., weight/ weight or area percent in all cases with respect to the major analyte.

5. Specificity/ selectivity

The terms specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method said to be selected. Since there are very few methods that respond to only one analyte, the term selectivity is more appropriate. The analyte should have no interference from other extraneous components and be well resolved from them. A representative chromatogram or profile should be generated and submitted to show that the extraneous peak either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

6. Ruggedness:

The ruggedness of an analytical method is the degree of reproducibility of test results Obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental Conditions that may differ but are still within the specified parameters of the assay. The testing of the ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

For the determination or ruggedness, the degree of reproducibility of test result is determined as a function of the assay variable. This reproducibility may be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness of the analytical method.

7. Robustness:

The concept of robustness of an analytical procedure has been defined by the ICH as "a Measure of its capacity to remain unaffected by small, but deliberate variations in method Parameters". A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters in HPLC technique may involve flow rate, column temperature, sample temperature, pH and mobile phase composition.

8. Stability:

To generate reproducible and reliable results, the samples, standards, and Reagents used for the HPLC method must be stable for a reasonable time (e.g., one

day, One week, and one month, depending on need). Therefore, a few hours of standard and sample solution suitability can required even for short (10 min) separation. When more than one sample is analyzed (multiple lots of one sample or samples from different storage conditions from a single lot), automated, overnight runs often are performed for better lab efficiency such practices add requirements for greater solution stability.

9. Limit of Detection:

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but the necessarily quantitated, under the stated experimental conditions.

- Based on visual Evaluation
- Based on the standard Deviation of the Blank
- Based on the calibration curve
- Based on signal-to-noise: A signal-to-noise ratio of 3 or 2:1 is acceptable

LOD may be expressed as

$$LOD = 3.3\sigma/s$$

Where, σ = the standard deviation of the response

S= the slope of the calibration curve

The slope S may be estimated from the calibration of the analyte.

10. Limit of quantitation:

Limit of quantitation is the lowest concentration of analyte in a sample that can be Determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantification limit are possible.

- Based on visual Evaluation
- Based on standard Deviation of the blink
- Based on the calibration curve
- Based on the signal-to-Noise Approach: A typical signal-to-Noise is 10:1

LOQ may be expressed as

$$LOQ = 10\sigma / s$$

Where, σ = standard deviation of the response

S= the slope of the calibration curve

2. LITERATURE REVIEW

1. Enjem Karunaker Reddy *et al.*; An isocratic, reversed phase-liquid-chromatographic method was developed for the quantitative determination of Azithromycin and Levofloxacin in combined-dosage form. A Waters Symmetry Shielde Rp18, (250*4.6*5µ) column with mobilephase containing water pH 9.2 adjusted with di- Potassium hydrogen Phosphate: Methanol in the ratio of (60: 40, v/v) was used.The flow rate was 1.0 mL/min, column temperature was 30°C and effluents were monitored at 285 nm. The retention times of Azithromycin and Levofloxacin were 5.001min and 3.232min, respectively. The correlation co-efficient for Azithromycin andLevofloxacin was found to be 0.99 and 0.99, respectively. The proposed method was validated with respect to linearity, accuracy,precision, specificity, and robustness. Recovery of Azithromycin and Levofloxacin in formulations was found to be in the range of97-103% and 97-103% respectively confirms the non-interferences of the excipients in the formulation. Due to its simplicity,rapidness and high precision. The method was successfully applied to the estimation of Azithromycin and Levofloxacin in combined dosage form.

2. Yun Nairu1 *et al.*; A RP-HPLC method was developed for the determination of Levofloxacin and Azithromycin in CM129 medium,in order to provide an Analysis Method to Experiment which Azithromycin promote Levofloxacin to penetrate biomembrane.Method The analytical column was Waters C18.The mobile phase consisted of which methanol monopotasium phosphate buffer=29∶71(pH=2.53).The flow rate was 1.0 ml/min,and the detector was set at UV 210 nm.Results : The calibration curve was linear in the range of 10～160 µg/ml,Correlation coefficient of the both two drug was 0.999 9 respectively,the average recovery of Levofloxacin

was 99.83% with RSD of 1.08% and the average recovery of Azithromycin was 100.34% with RSD of 0.47%.Conclusion : This method has been applied to the determinate the concentrations of Levofloxacin and Azithromycin simultaneously.

3. **M. Rachidi *et al.*;** Azithromycin (AZT), an antibiotic belonging to the family of macrolides, can be analyzed by a new spectrophotometric method based on the formation of an ion pair between this drug and an inorganic complex of (Mo(V)–thiocyanate) followed by its extraction with dichloroethane. This ion-association complex shows an orange color and exhibits a maximum absorbance at 469 nm. The experimental conditions of the reaction were studied and optimized. The calibration graph was linear (r=0.9996) over the range 10^{-6} M–10^{-5} M of AZT. This simple and validated method has been successfully applied to the determination of azithromycin in pharmaceutical formulations with a mean relative standard deviation of 1.07% and mean recovery of 99.66%. The common excipients present in azithromycin formulations did not interfere in its determination. This new spectophotometric method has been applied successfully to illustrate the dissolution profiles of original tablets and generic compounds; hence, it could be employed in routine quality control of azithromycin in pharmaceutical dosage forms..

4. S.C. Arora *et al.*; Azithromycin Dihydrate (Poorly water soluble drug), when prepared as solid dispersion showed improved solubility and dissolution. So the main purpose of this investigation was to increase the solubility and dissolution rate of Azithromycin Dihydrate by the preparation of its solid dispersion with urea using solvent evaporation method. Physical mixtures and solid dispersions of Azithromycin Dihydrate were prepared by using urea as water-soluble carrier in various proportions (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7 by weight), by employing

solvent evaporation method. The drug release profile was studied and it was found that the dissolution rate and the dissolution parameters of the drug from the physical mixture as well as solid dispersion were higher than those of the intact drug. FT- IR spectra revealed no chemical incompatibility between drug and urea. Drug-polymer interactions were investigated using differential scanning calorimetry (DSC) and Powder X-Ray Diffraction (PXRD).

5. Makarand Avhad *et al.*; An accurate, specific and precise UV spectrophotometric method was developed for the simultaneousdetermination of levofloxacin (LVF) and ambroxol (AMB) in pharmaceutical dosage forms. The method involves formation ofQ-absorbance equation at 219 (isoabsorptive point) and at 287 nm, using distilled water as a solvent. The linearity for bothlevofloxacin and ambroxol was in the range of 2-14 µg/ml and 5-35 µg/ml respectively. The % recovery was found to be 100-101% and 101-102% for levofloxacin and ambroxol respectively indicating proposed method is accurate and precise forsimultaneous estimation of levofloxacin and ambroxol in tablets.

6.S. Schulte *et al.*; A simple, accurate, sensitive, and precise reversed-phase (RP) high-performance liquid chromatographic (HPLC) method with fluorescence detection allowing the sensitive and specific quantitation of the newer fluoroquinolones levofloxacin and moxifloxacin is described. Moxifloxacin is used as the internal standard for the determination of levofloxacin and vice versa. A single-step liquid-liquid extraction from human plasma is sufficient for both quinolones. The method is linear from 0.1 to 15 µg/mL and 0.2 to 7 µg/mL for levofloxacin and moxifloxacin, respectively, covering the clinically relevant plasma concentration range. The limits of quantitation are 0.05 µg/mL (levofloxacin) and

0.2 µg/mL (moxifloxacin). The method is successfully applied to plasma drug level monitoring in a volunteer receiving single therapeutic doses of levofloxacin or moxifloxacin at two different occasions.

7. M. Senthil Raja1 *et al.*; A simple reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of Azithromycin and Ambroxol Hydrochloride in combined dosage form. Theseparation was carried out using a mobile phase consisting of acetonitrile and mono basic potassium phosphate buffer of pH 8.5 in the ratio of 65:35 v/v. The column used was C18 phenomenex Gemini 5m, 250cm x 4.6mm id with flow rate of2ml/min using PDA detection at 220nm. The described method was linear over a concentration range of 96-145mg/mland 80-125mg/ml for the assay of Azithromycin and Ambroxol Hydrochloride respectively. The retention times of Ambroxol and Azithromycin were found to be 3.7min and 6.1min respectively. Results of analysis were validatedstatistically and by recovery studies. The limit of quantification (LOQ) for Azithromycin and Ambroxol Hydrochloridewere found to be 96.7mg/ml and 8.35mg/ml respectively. Then the limit of detection (LOD) for Azithromycin and Ambroxol Hydrochloride were found to be 31.91 mg/ml and 2.75 mg/ml respectively.

8. Vishal Shah *et al.*; A novel, simple, accurate, sensitive, reproducible, economical spectroscopic methodwas developed and validated for the determination of Azithromycin dihydrate andCefixime trihydrate in combined dosage form. Second order derivative spectroscopymethod is adopted to eliminate spectral interference. The method obeys Beer's Law inconcentration ranges of 10-40 ppm for Cefixime trihydrate and 25-100 ppm of Azithromycin dihydrate. The method was validated

for linearity, accuracy andprecision as per ICH guidelines. The zero crossing point for Azithromycin dihydrateand Cefixime trihydrate was 328 nm and 231.6 nm, respectively in water. The LODand LOQ value were found to be 0.13 and 0.38 ppm for Cefixime trihydrate and 0.80and 2.44 ppm for Azithromycin dihydrate respectively.

3. DRUG PROFILE

3.1 LEVOFLOXACIN

It inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase.

Structure:

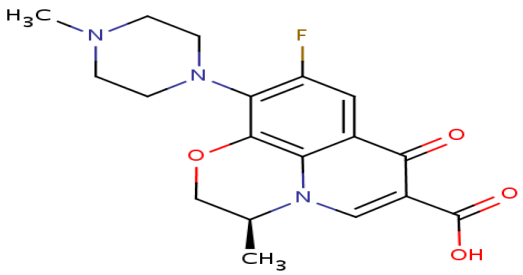


Fig no:3 Structure of Levofloxacin

IUPAC Name : (2S)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.0^{5,13}]trideca-5(13),6,8,11-tetraene-11-carboxylic acid

Molecular formula : $C_{18}H_{20}FN_3O_4$

Molecular Weight : 361.3675 /mol

Solubility : *slightly soluble in water, soluble in glacial acetic acid.*

Pka : 5.45

Mechanism of action:

Levofloxacin inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase. Levofloxacin, like other fluoroquinolones, inhibits the A subunits of DNA gyrase, two subunits encoded by the gyrA gene. This results in strand breakage on a bacterial chromosome, supercoiling, and resealing; DNA replication and transcription is inhibited.

Indications: For the treatment of bacterial conjunctivitis caused by susceptible strains of the following organisms: *Corynebacterium* species, *Staphylococus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus* (Groups C/F/G), Viridans group streptococci, *Acinetobacter lwoffii*, *Haemophilus influenzae*, *Serratia marcescens*..

Absorption: Absorption of ofloxacin after single or multiple doses of 200 to 400 mg is predictable, and the amount of drug absorbed increases proportionately with the dose.

Protein binding: 24-38% (to plasma proteins)

Metabolism: Mainly excreted as unchanged drug (87%); undergoes limited metabolism in humans.

Pharmacodynamics: Levofloxacin, a fluoroquinolone antiinfective, is the optically active L-isomer of ofloxacin. Levofloxacin is used to treat bacterial conjunctivitis, sinusitis, chronic bronchitis, community-acquired pneumonia and pneumonia caused by penicillin-resistant strains of *Streptococcus pneumoniae*, skin and skin structure infections, complicated urinary tract infections and acute pyelonephritis.

Half Life: 6-8 hours

Route of elimination:

Mainly excreted as unchanged drug in the urine.

Toxicity:

Side effects include disorientation, dizziness, drowsiness, hot and cold flashes, nausea, slurring of speech, swelling and numbness in the face

3.2 AZITHROMYCIN

It binds to the 50S subunit of the 70S bacterial ribosomes, and therefore inhibits RNA-dependent protein synthesis in bacterial cells.

Structure:

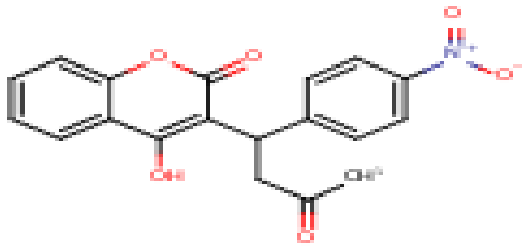


Fig no:4 Stuctue of Azithromycin

IUPAC Name: (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-11-{[(2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-2-ethyl-3,4,10-trihydroxy-13-{[(2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one

Molecular formula : $C_{38}H_{72}N_2O_{12}$

Molecular Weight : 748.9845 gm/mol

Solubility : Freely soluble in methanol, slightly soluble in 95%ethonol, very slightly soluble in water

Pka : 8.74

Mechanism of action:

Azithromycin binds to the 50S subunit of the 70S bacterial ribosomes, and therefore inhibits RNA-dependent protein synthesis in bacterial cells..

Absorption: Bioavailability is 37% following oral administration. Absorption is not affected by food. Azithromycin is extensively distributed in tissues

with tissue concentrations reaching up to 50 times greater than plasma concentrations. Drug becomes concentrated within macrophages and polymorphonucleocytes giving it good activity against Chlamydia trachomatis.

Protein binding: Serum protein binding is variable in the concentration range approximating human exposure, decreasing from 51% at 0.02 µg/mL to 7% at 2 µg/mL.

Metabolism: Hepatic. In vitro and in vivo studies to assess the metabolism of azithromycin have not been performed.

Pharmacodynamics:

Azithromycin, a semisynthetic antibiotic belonging to the macrolide subgroup of azalides, is used to treat STDs due to chlamydia and gonorrhea, community-acquired pneumonia, pelvic inflammatory disease, pediatric otitis media and pharyngitis, and *Mycobacterium avium* complex (MAC) in patients with advanced HIV disease. Similar in structure to erythromycin. azithromycin reaches higher intracellular concentrations than erythromycin, increasing its efficacy and duration of action.

Route of elimination:

Biliary excretion of azithromycin, predominantly as unchanged drug, is a major route of elimination.

Half life: 68 hours

Toxicity: Potentially serious side effects of angioedema and cholestatic jaundice were reported.

AIM AND OBJECTIVE

4. 1 AIM & OBJECTIVE

Existing literature reveals that Levofloxacin and Azithromycin can be analyzed by UV detection, HPTLC, HPLC individually and combination with other drugs in bulk material and pharmaceutical forms.

A comprehensive, validated and simple analytical simultaneous method development and validation of Levofloxacin and Azithromycin is, therefore, crucial. No economic, simple and precise HPLC method was there for simultaneous estimation of Levofloxacin and Azithromycin in bulk and pharmaceutical dosage forms. Therefore, in proposed project a successful attempt has been made to develop, simple, Accurate, and economic methods for analysis of Levofloxacin and Azithromycin tablets validated.

OBJECTIVE

The objective of the present work is to development and validates a HPLC method development and validation Levofloxacin and Azithromycin of tablets. To be employed in routine analysis.In the method development of Levofloxacin and Azithromycin we have decided to carry out our project work by incorporating the Reverse phase High performance Liquid chromatography (HPLC).Then the developed method will be validated according to ICH guidelines for its various parameters.

PLAN OF WORK

In order to develop a simple, reliable and an accurate method development and validation of Levofloxacin and Azithromycin in pharmaceutical dosage form by Reverse phase HPLC and validate the method for its repeatability and reproducibility

Plan of the proposed work includes the following steps:

- Selection of drug and literature survey.
- Solubility studies and optimization of conditions.
- Analytical method(s) development using HPLC etc.,
- Assay of the drugs(s) in marketed formulations using the proposed method(s).
- Procurement of raw materials.
- Establishment of system suitability parameters.
- Trails for the method development of Levofloxacin and Azithromycin Setting of the optimized method.
- Validation of the optimized method for Levofloxacin and Azithromycin

Validation parameters include

- ❖ System suitability
- ❖ Specificity

- precision
- Linearity
- Accuracy
- Range
- Ruggedness
- Robustness
- Assay

MATERIALS AND METHODS

6.1 INSTRUMENTS/EQUIPMENT USED

Instruments:

- WATERS HPLC, Model: Aglient 2695, Photo diode array detector (PDA), with an automated sample injector. The output signal was monitored and integrated using Empower 2 software.ELIPSE C8 (150mm*4.6, 5 µm, Make: Waters) column was used for separations.

Table 7 : List of Equipments

S.NO	Equipment's	Model	Company
1	Electronic Balance	ER200A	ASCOSET
2	Ultra-Sonicator	SE60US	ENERTECH
3	Heating Mantle	BTI	BIO TECHNICS INDIA
4	Thermal oven	---------	NARANG
5	pH Meter	AD102U	ADWA
6	Filter Paper 0.45 microns	---------	MILLI PORE

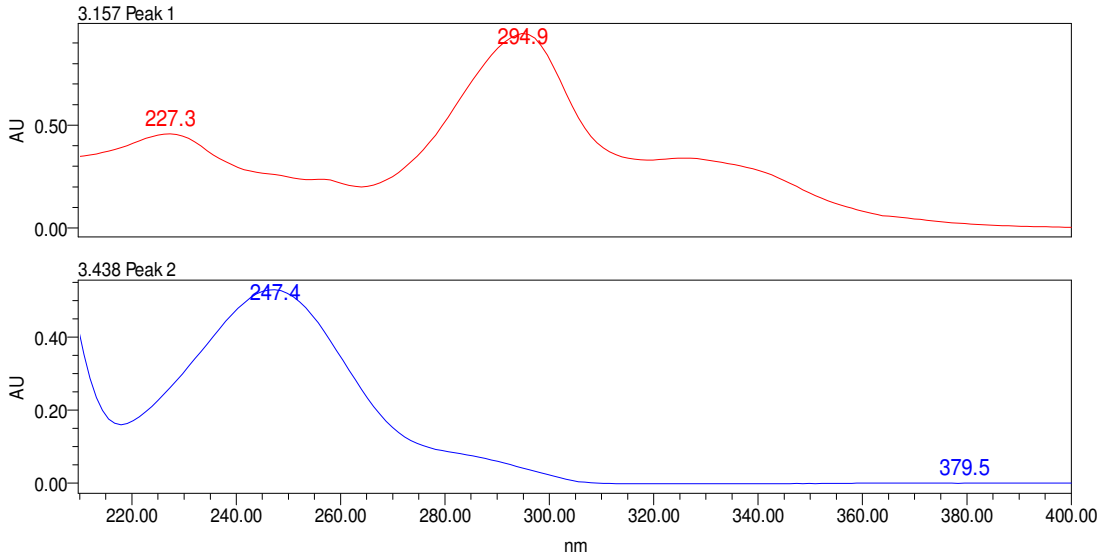
6.2 CHEMICALS AND REAGENTS USED

Table no.8: List of chemicals and reagents used

S. No.	Chemicals/standards and reagents	Grade	Make
1	KH_2PO_4	AR	Finar
2	Methanol	HPLC	Merck
3	Water	HPLC	Loba Chemi
4	DiPotassium hydrogen phosphate	AR	Dr. Reddy's
5	Levofloxacin	NA	Dr. Reddy's
6	Azithromycin	NA	Dr. Reddy's

5.3 METHOD DEVELOPMENT

The UV spectrums of Levofloxacin and Azithromycin under these mobile phase conditions were shown below and from these spectrums, Lambda Max 265 nm were observed**.**



METHOD DEVELOPMENT TRAILS:

Trial-1

Mobile Phase : 0.1%OPA: Methanol (600:400)

Column : Agilent ZorbaxC18, 250x4.6, 5µ

Flow Rate : 1.0ml/min

Injection Volume : 10 µl

Column Temperature : 30˚C

Detector : 265nm

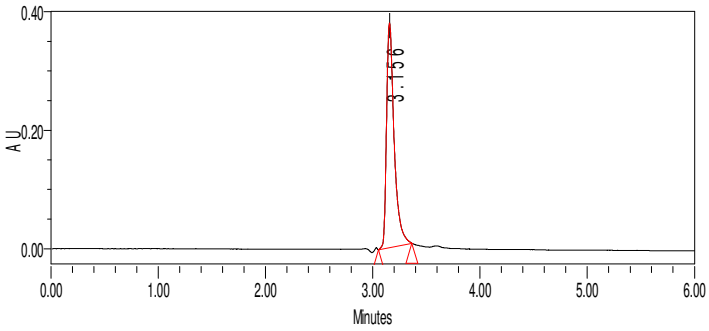


Fig. no.8: Typical chromatogram of trail 1

	Name	Retention Time	Area	USP Tailing	USP Plate Count
1	Levofloxacin	3.156	1863337	1.61	10517

Observation: There is no second peak

Reason: May low Concentration

May be insoluble of Drug

Trial-2

Mobile Phase : 0.1%OPA: Methanol (600:400)

Column : Agilent ZorbaxC18, 250x4.6, 5µ

Flow Rate : 1.0ml/min

Injection Volume : 10 µl

Column Temperature : 30˚C

Detector : 265nm

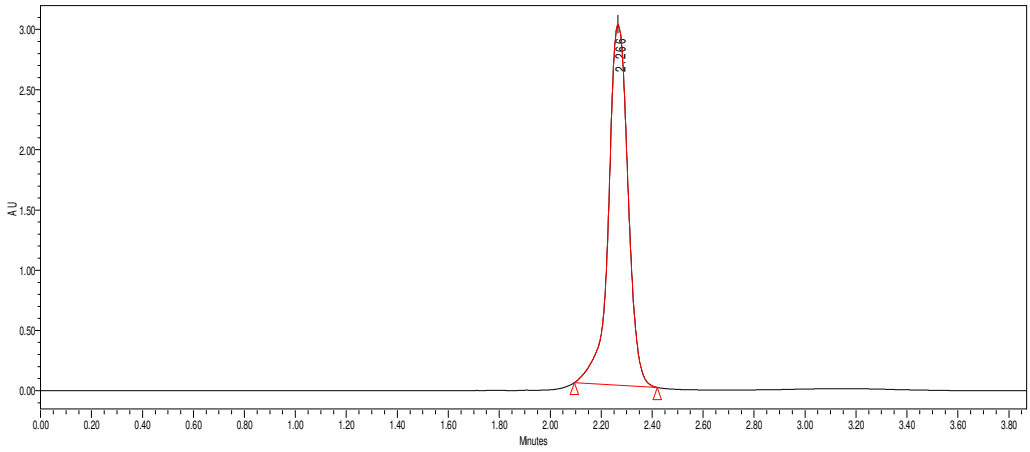


Fig. no. 9: Typical chromatogram of trail 2

	Name	Retention Time	Area	USP Tailing	USP Plate Count
1	Levofloxacin	2.266	16013611	0.92	5191

Observation: Even there is no second peak

Reason: May be Mobile Phase Ph problem

Trial-3

Mobile Phase : KH2PO4: Methanol (800:200)

Column : Agilent ZorbaxC18, 250x4.6, 5µ

Flow Rate : 1.0ml/min

Injection Volume : 10 µl

Column Temperature : 30˚C

Detector : 265nm

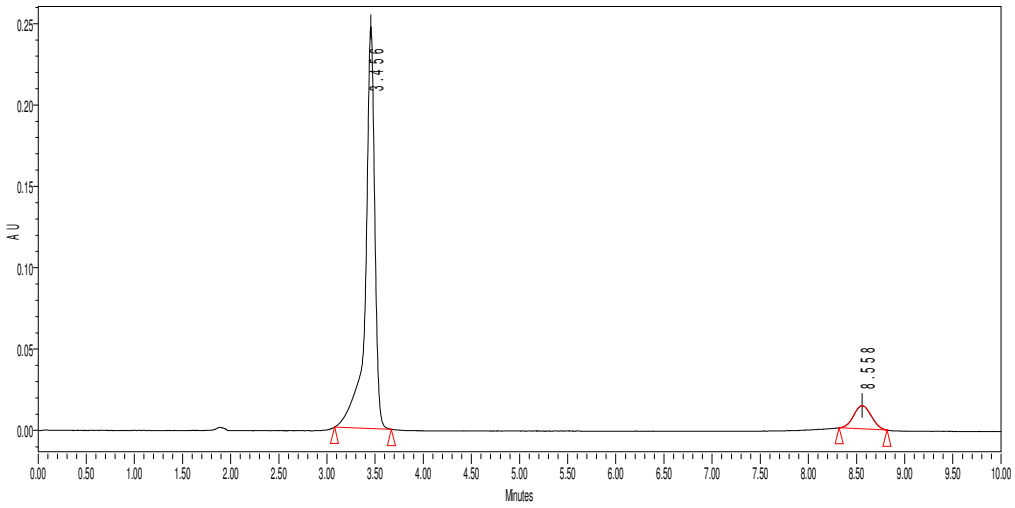


Fig. no.10: Typical chromatogram of trail 3

	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Levofloxacin	3.456	1655193		0.72	8570
2	Azithromycin	8.558	182768	20.25	1.07	10061

Observation: Yes we got two peaks with good resolution but second peak response is very poor.

Trial-4

Mobile Phase : KH2PO4: Methanol (600:400)

Column : Agilent ZorbaxC18, 250x4.6, 5µ

Flow Rate : 1.0ml/min

Injection Volume : 10 µl

Column Temperature : 30˚C

Detector : 265nm

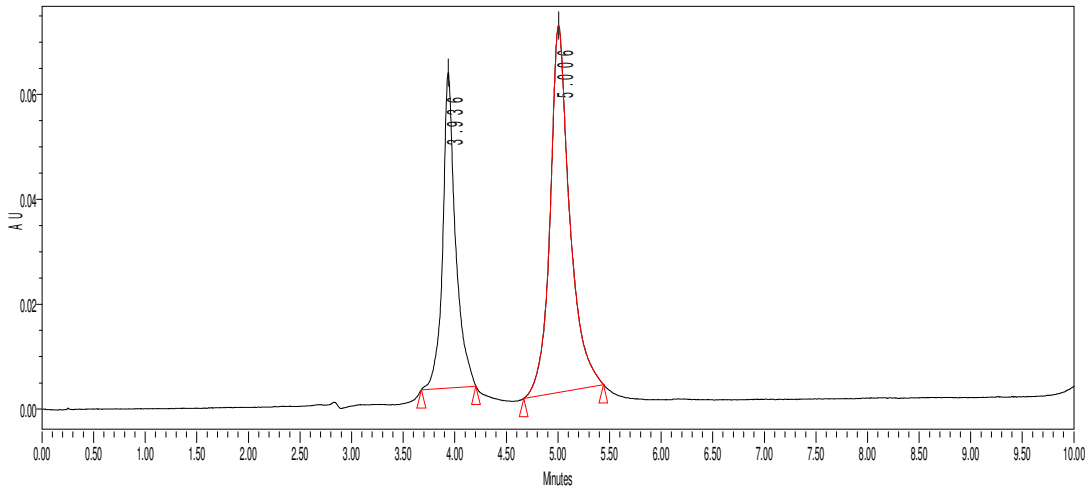


Fig. no.11: Typical chromatogram of trail 4

	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Levofloxacin	3.936	520470		1.21	5853
2	Azithromycin	5.006	928473	3.69	1.20	3726

Observation: Yes we got two peaks but low resolution

Reason: May be buffer volume low in Mobile Phase

Trial-5

Mobile Phase : K2HPO4: Methanol (400:600)

Column : Agilent ZorbaxC18, 250x4.6, 5µ

Flow Rate : 1.0ml/min

Injection Volume : 10 µl

Column Temperature : 30˚C

Detector : 265nm

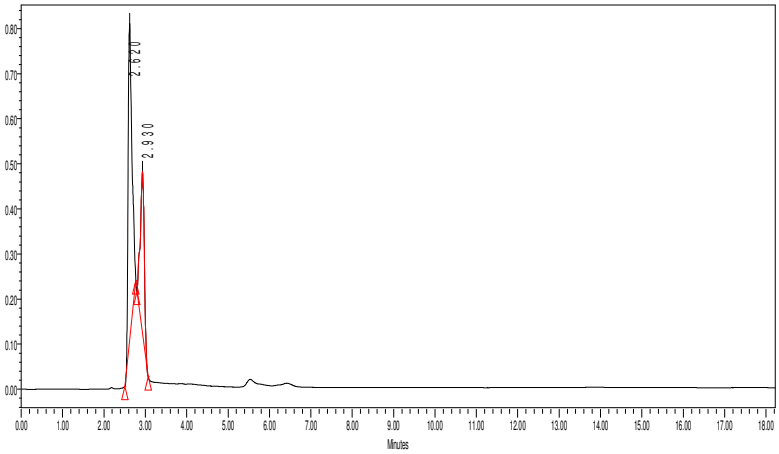


Fig. no. 12: Typical chromatogram of trail 5

	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Levofloxacin	2.620	4512330		1.36	3875
2	Azithromycin	2.930	2417939	1.71	0.95	4874

Observation: Yes we got two peaks but low resolution

Reason: May be buffer volume low in Mobile Phase

Trial-6

Mobile Phase : K2HPO4: Methanol (400:600)

Column : Agilent ZorbaxC18, 250x4.6, 5µ

Flow Rate : 1.0ml/min

Injection Volume : 10 µl

Column Temperature : 30˚C

Detector : 265nm

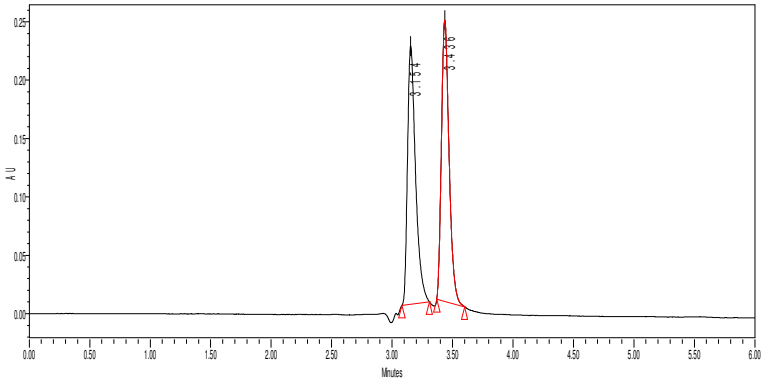


Fig. no. 13: Typical chromatogram of trail 6

	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Levofloxacin	3.154	1058576	2.33	1.35	14206
2	Azithromycin	3.436	1022805		1.55	11075

Observation:

RT was found to be good and the peak symmetry of both drugs were good. And the resolution theoretical plate count and tailing were within the limits and it is used for validation of the method.

OPTIMIZED METHOD

Chomatographic parameters:

Mobile Phase **:** K2HPO4: Methanol (400:600)

Column **:** Agilent ZorbaxC18, 250x4.6, 5µ

Flow Rate **:** 1.0ml/min

Injection Volume **:** 10 µl

Column Temperature **:** 30˚C

Detector **:** 265nm

Procedure:

Inject 10µL of standard, sample into chromatographic system and measure the areas for the Levofloxacin and Azithromycin peaks and calculate the % assay by using the formula

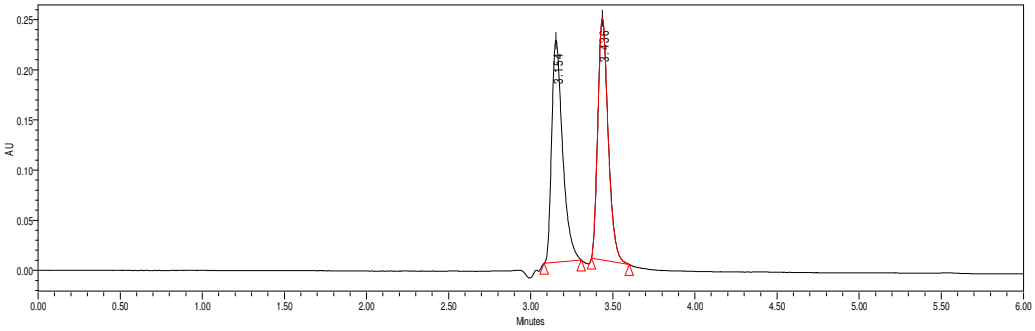


Fig. no.14: Chromatogram for optimized method

	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Levofloxacin	3.154	1058576	2.33	1.35	14206
2	Azithromycin	3.436	1022805		1.55	11075

Observation:

Peeks are well separated all the parameters are within the limits. For quantitative analytical purpose wavelength was set at 265 nm, which provided better reproducibility.

PREPARATION OF MOBILE PHASE:

Transfer 500ml of HPLC water into 500ml of beaker and DiPotassium hydrogen phosphate adjust pH 3.5 using O-phosphoric acid. Transfer the above solution 400mlof K2HPO4, 600ml of Methanol is used as mobile phase. They are mixed and sonicated for 20min.

PREPARATION OF THE LEVOFLOXACIN AND AZITHROMYCIN STANDARD AND SAMPLE SOLUTION:

PREPARATION OF STANDARD SOLUTION:

Accurately weigh and transfer 100mg of Levofloxacin and Azithromycin into 100ml of volumetric flask and add 10ml of Methanol and sonicate 10min (or) shake 5min and make with water. Transfers the above solution into 5ml into 50ml volumetric flask dilute to volume with water.

PREPARATION OF SAMPLE STOCK SOLUTION:

Commercially available 20 tablets ware weighed and powdered the powdered equivalent to the 500mg of Levofloxacin and Azithromycin of active ingredients were transfer into a 100ml of volumetric flask and add 10ml of Methanol and sonicate 20min (or) shake 10min and makeup with water. Transfers above solution 5ml into 50ml of the volumetric flask dilute the volume with Methanol. And the solution was filtered through 0.45µm filter before injecting into HPLC system.

6.4 ASSAY RESULT FOR FORMULATION

Label contains: Each film coated tablet contains

Levofloxacin - 500mg.

Azithromycin -500 mg.

Average weight of each tablet is 1383.0mg

Purity of working standards:

1. Levofloxacin: %purity-99.1%

2. Azithromycin: % purity –99.7%

Sample preparation:

10 tablets were weighed and crushed, from the powdered tablets, weighed accurately about 500mg(500mg Levofloxacin and 500mg Azithromycin) into a 100 ml volumetric flask and 50 ml of mobile phase was added. The mixture was subjected to sonication for 20 min with intermediate shaking for complete extraction of drugs. Filtered and cooled to room temperature and solution was made up to mark with mobile phase. From the above solution 5 mL is taken and further diluted in 25 ml volumetric flasks with mobile phase. To acquire a concentration of 500mg Levofloxacin and 500mg Azithromycin.

Standard preparation:

Accurately weighed quantity of 500mg Levofloxacin and 500mg Azithromycin was taken in a 100 ml volumetric flask and 50 ml of mobile phase

was added. The mixture was subjected to sonication for 20 min with intermediate shaking for complete extraction of drugs. Filtered and cooled to room temperature and solution was made up to mark with mobile phase. From the above solution 5 ml is taken and further diluted in 25 mL volumetric flasks with mobile phase.To acquire a concentration of 500mg Levofloxacin and 500mg Azithromycin.

Procedure:

Separately injected both the standard (2 injections) and sample preparations (2 injections) into the chromatographic system and recorded the peak area responses.

% percentage content =

$$\frac{\text{Sample area} \times \text{Sample dilution} \times \text{Avg weight} \times \text{standard weight} \times \text{purity of working standard} \times 100}{\text{Standard area} \times \text{standard dilution} \times \text{label claim} \times \text{sample weight}}$$

6.5 METHOD VALIDATION

1. SYSTEM SUITABILITY:

Tailing factor for the peaks due to Levofloxacin and Azithromycin in standard solution should not be more than 2.0.Theoretical plates for the Levofloxacin and Azithromycin peaks in standard solution should not be less than 2000.

2. SPECIFICITY:

Solution of standard, sample, blank and placebo were prepared as per test procedure and injected into the HPLC system.

Acceptance criteria:

Chromatogram of standard and sample should be identical with near Retention time.

Blank interference:

A study to establish the interference of blank was conducted. Diluent was injected into HPLC system as per the test procedure.

Acceptance criteria:

Chromatogram of blank should not show any peak at the retention time of analytepeak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

3. LINEARITY

Prepare a series of standard solutions and inject into HPLC system. Plot the graph of standard versus the actual concentration in µg/ml and determine the coefficient of correlation and basis for 100% response.

Acceptance criteria:

Linearity regression coefficient of average peak area response of replicate injections plotted against respective concentration should not be less than 0.999. The % y-intercept as obtained from the linearity data (without extrapolation through origin 0, 0) should be within ±2.0.

Statistical Evaluation:

A graph between the concentration and the average area was plotted. Points for linearity were observed. Using the method of least squares, a line of best fit was taken and the correlation Coefficient, slope and, y-intercept were calculated.

4. PRECISION:

Preparation of sample:

- Transfer the 200.5mg of sample into a 100ml of volume at flask and add 10ml of water and 10ml of Methanol and sonicate 20min and makeup with water. Transfer the above solution into 5ml into 50ml volume metric flask dilute to the volume with water.

- The method precision parameters were evaluated from sample chromatograms obtained, by calculating the % RSD of peek areas from 6 replicate injection.

Acceptance criteria: The injection reproducibility requirements are met if the %RSD for peak areas is not more than 2.0 and for retention times is not more than 2.0.

5. RECOVERY/ACCURACY

Recovery study can be performed in the concentration range of 80% to 120% of the target concentration of the test. Minimum 3 concentrations are recommended.

Acceptance criteria:

The average percentage recovery was between 98-102% and Relative standard deviation of these recovery concentrations was less than 2%.

6. LIMIT OF DETECTION

The sensitivity of measurement of Levofloxacin and Azithromycin by use of proposed method was estimated in terms of the limit of detection (LOD). The LOD was calculated by the use of signal to noise ratio. In order to estimate the LOD value, the blank sample was injected six times and peak area of this blank was calculated as noice level. The LOD was calculated as three times the noise level.

$$LOD = 3.3\ \sigma / S$$

Where,

σ = standard deviation of intercepts of calibration curves

S = mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

7. LIMIT OF QUANTITATION:

The sensitivity of measurement of Levofloxacin and Azithromycin by the use of proposed method was estimated in terms of limit of quantitation (LOQ). The LOQ was calculated by the use of signal to noise ratio. In order to estimate the LOQ value, the blank sample was injected six times and the peak area of this blank was calculated at noise level. The LOQ was calculated as ten times the noise value gave the LOQ.

$$LOQ = 10\ \sigma / S$$

Where,

σ = standard deviation of intercepts of calibration curves

S = mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

8. ROBUSTNESS:

Effect of variation in flow rate:

Prepare the system suitability solution as per the test method and inject into the HPLC system with ±0.2 ml of the method flow. Evaluate the system suitability values as required by the test method for both flow rates. Actual flow rate was 1.0

ml/min and it was changed to 0.8ml/min and 1.2ml/min and inject into HPLC and system suitability was checked.

Effect of variation in wavelength:

Prepare the system suitability solution as per the test method and injected into the HPLC with ±2nm variation in wavelength. Evaluate the system suitability values as required by the test method for both wavelengths.

7. RESULTS AND DISCUSSION

1. SYSTEM SUITABILITY:

Table 9: System suitability data of Levofloxacin and Azithromycin

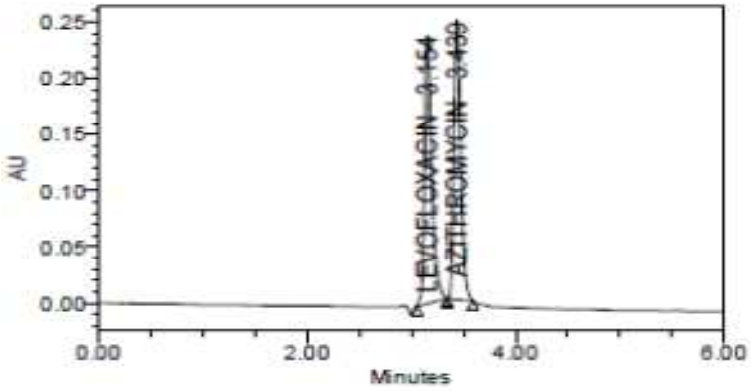
parameter	Levofloxacin	Azithromycin	Acceptance criteria
Retention time	3.154	3.436	+-10
Theoretical plates	14206	11075	>2500
Tailing factor	1.35	1.55	<2.00
% RSD	0.3	0.2	<2.00

Table 10 : Standard Results of Levofloxacin

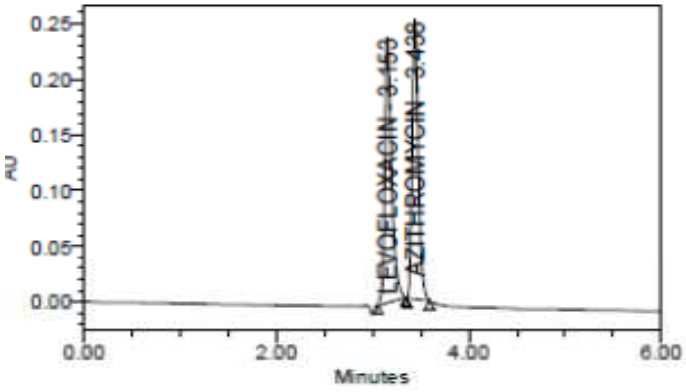
S.no	Sampl name	RT	Area	USP plate count	USP tailing
1.	Injection1	3.154	1136532	11143	1.49
2.	Injection 2	3.153	1142370	10970	1.51
3.	Injection 3	3.151	1134603	11208	1.47
4.	Injection 4	3.149	1139792	11201	1.50
5.	Injection 5	3.149	1132720	11369	1.48

Table 11 : Standard Results of Azithromycin

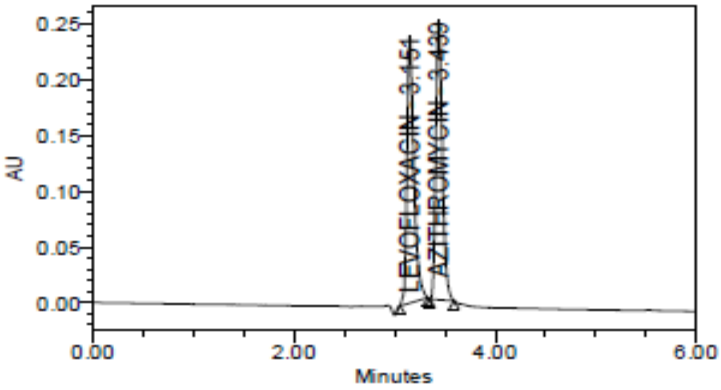
S.no	Sample name	RT	Area	USP plate count	USP tailing
1.	Injection 1	3.439	1110718	13998	1.30
2.	Injection 2	3.438	1115155	14170	1.31
3.	Injection 3	3.439	1115740	14068	1.31
4.	Injection 4	3.435	1111439	14266	1.31
5.	Injection 5	3.434	1112680	14175	1.30



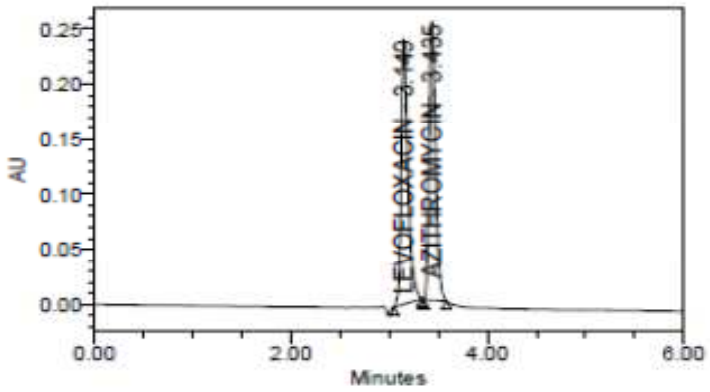
typical Chromatogram of Standard-2; Injection-1



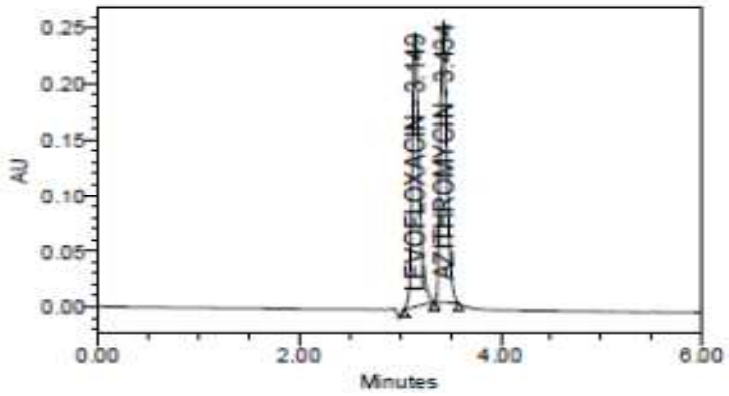
typical Chromatogram of Standard-2; Injection-2



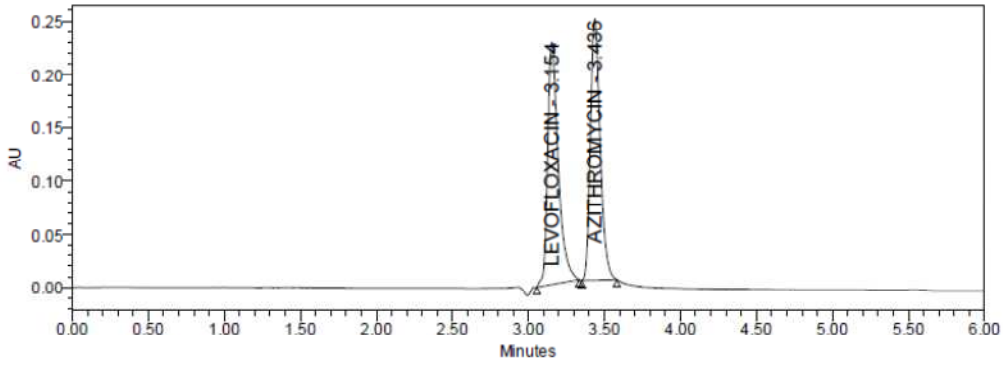
typical Chromatogram of Standard-2; Injection-3



typical Chromatogram of Standard-2; Injection-4



typical Chromatogram of Standard-2; Injection 5



Typical Chromatogram of Standard-1

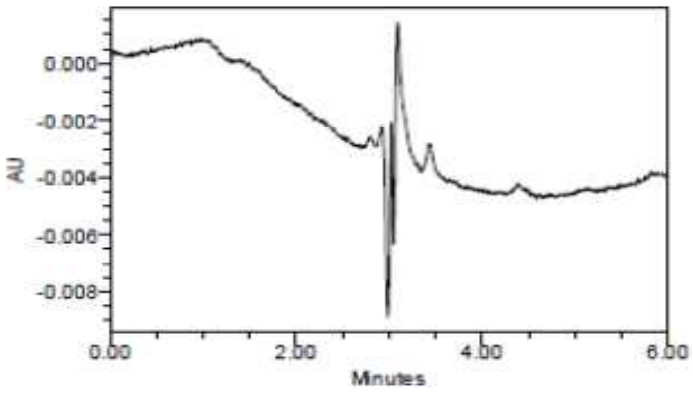
RESULT

Results of system suitability study are summarized in the above table. Six consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis.

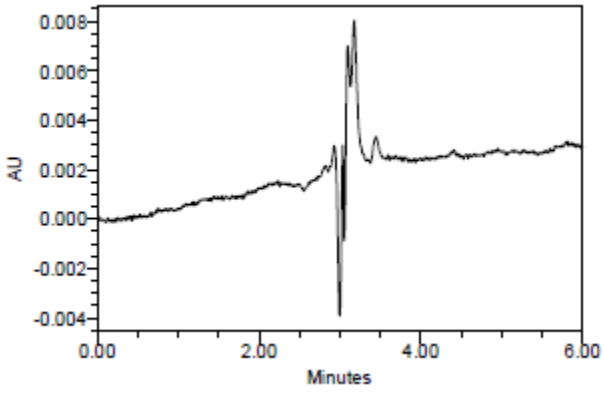
2. SPECIFICITY:

Table 12 : Specificity data for Levofloxacin and Azithromycin

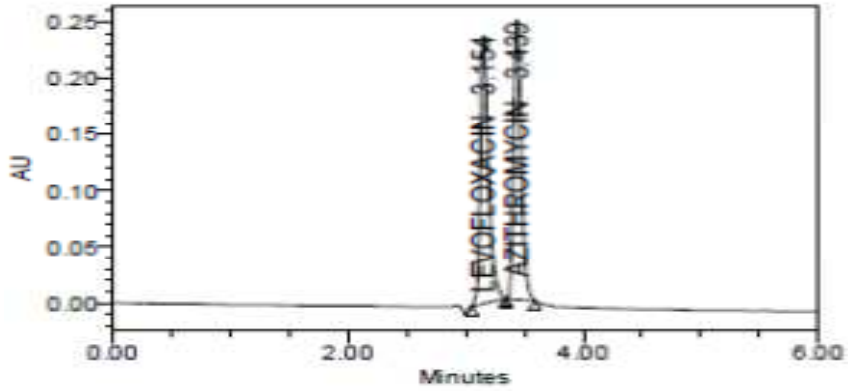
S no	Sample name	Levofloxacin area	Rt	Azithromycin Area	Rt
1	Standard	1136532	3.154	1110718	3.439
2	Sample	1132720	3.149	1112680	3.434
3	Blank	-	-	-	-
4	Placebo	-	-	-	-



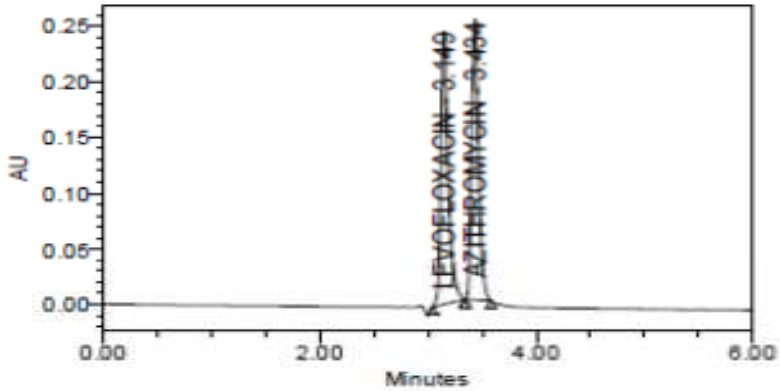
Typical chromatogram of the blank



Typical chromatogram of the Placebo



chromatogram representing specificity of standard



chromatogram representing specificity of sample

RESULT

Chromatograms explain that retention time for standard, sample and commercial product of Levofloxacin and Azithromycin are same. This proves that, excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So the method is highly selective.

3. ACCURACY:

Table 13 : Accuracy data for Levofloxacin

S.NO	Accuracy level	injecton	Sample area	RT
1	50%	1	568379	3.144
		2	568966	3.135
		3	568795	3.143
		4	568355	3.140
		5	568610	3.140
		6	568330	3.147
2	100%	1	1132033	3.137
		2	1130164	3.140
		3	1130359	3.150
3	150%	1	1707811	3.139
		2	1701236	3.150
		3	1708003	3.154
		4	1700653	3.146
		5	1709110	3.149
		6	1702553	3.154

Table 14 : Accuracy (%recovery) results of Levofloxacin

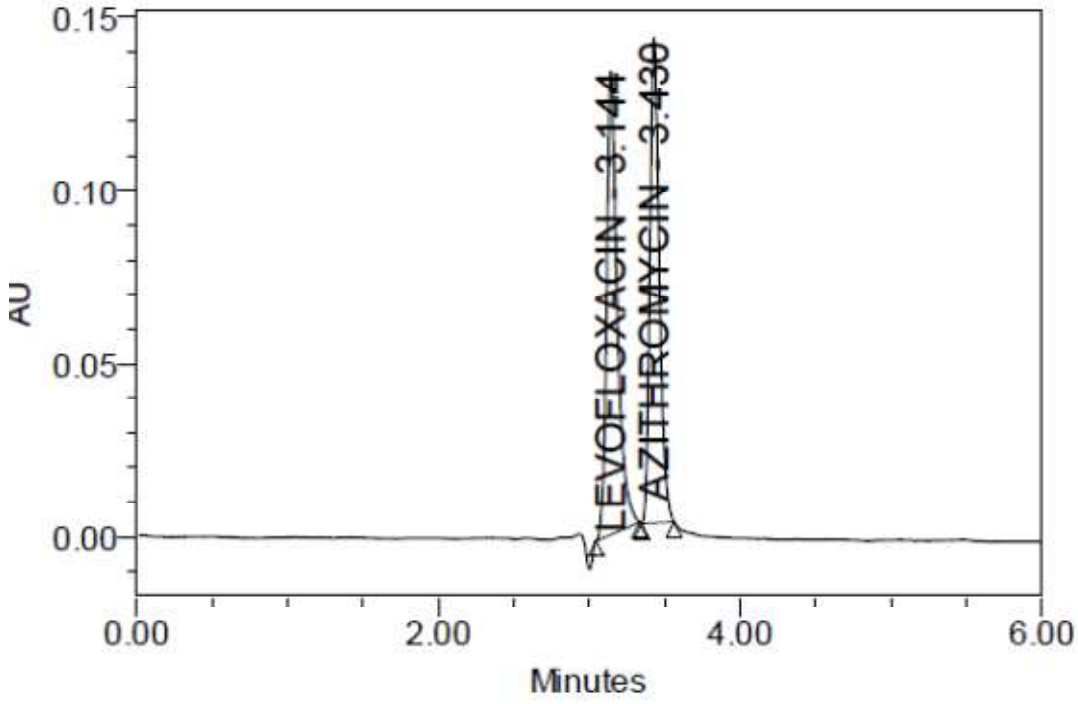
S.NO	Accuracy level	Sample name	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
1	50%	1	691.50	49.500	49.53	100	100
		2	691.50	49.500	49.58	100	
		3	691.50	49.500	49.57	100	
		4	691.50	49.500	49.53	100	
		5	691.50	49.500	49.55	100	
		6	691.50	49.500	49.53	100	
2	100%	1	1383.0	99.000	98.65	100	100
		2	1383.0	99.000	98.49	99	
		3	1383.0	99.000	98.50	99	
3	150%	1	2074.50	148.500	148.82	100	100
		2	2074.50	148.500	148.25	100	
		3	2074.50	148.500	148.84	100	
		4	2074.50	148.500	148.20	100	
		5	2074.50	148.500	148.94	100	
		6	2074.50	148.500	148.37	100	

Table : Accuracy data for Azithromycin

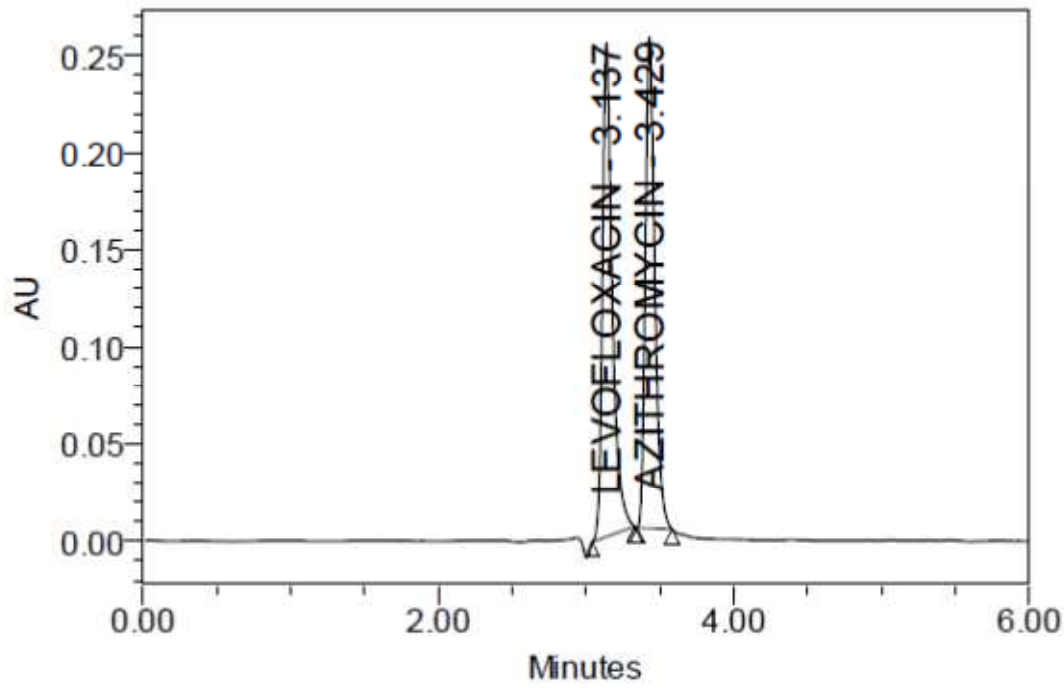
S.NO	Accuracy level	Sample name	Sampl area	RT
1	50%	1	556537	3.430
		2	556271	3.423
		3	556932	3.429
		4	556794	3.424
		5	556958	3.426
		6	556505	3.432
2	100%	1	1115710	3.429
		2	1114936	3.430
		3	1113708	3.439
3	150%	1	1662149	3.432
		2	1663514	3.443
		3	1665465	3.447
		4	1660861	3.439
		5	1669800	3.443
		6	1662124	3.447

Table 16: Accuracy (%recovery) results of Azithromycin

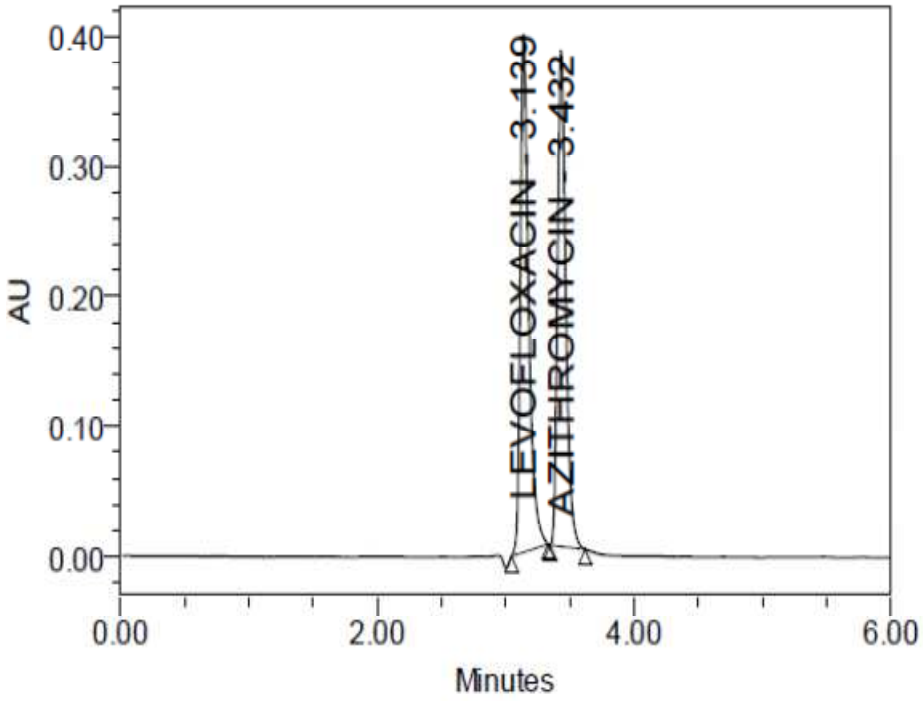
S.NO	Accuracy level	Sample name	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
1	50%	1	691.50	50.000	49.85	100	100
		2	691.50	50.000	49.82	100	
		3	691.50	50.000	49.88	100	
		4	691.50	50.000	49.87	100	
		5	691.50	50.000	49.88	100	
		6	691.50	50.000	49.84	100	
2	100%	1	1383.00	100.000	99.93	100	100
		2	1383.00	100.000	99.86	100	
		3	1383.00	100.000	99.75	100	
3	150%	1	2074.50	150.000	148.87	99	100
		2	2074.50	150.000	148.99	99	
		3	2074.50	150.000	149.17	99	
		4	2074.50	150.000	148.76	99	
		5	2074.50	150.000	149.56	100	
		6	2074.50	150.000	148.87	99	



Typical chromatogram for Accuracy 50 %



Typical chromatogram for Accuracy 100 %



Typical chromatogram for Accuracy 150 %

RESULT

Results of accuracy study are presented in the above table. The measured value was obtained by recovery test. Spiked amount of both the drug were compared against the recovery amount.

% Recovery was 100.00% for Levofloxacin and 100.00% for Azithromycin. All the results indicate that the method is highly accurate**.**

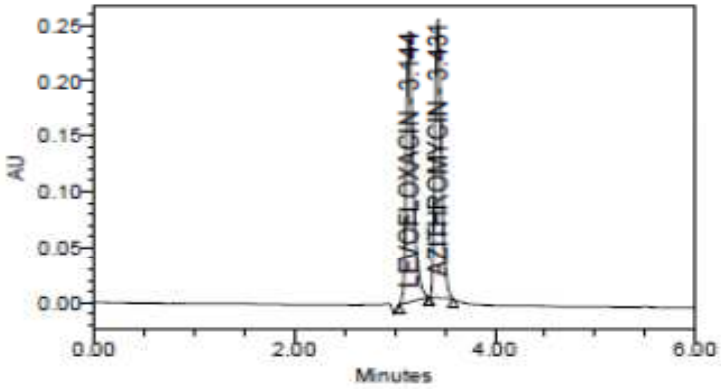
4.**PRECISION:**

Table 17: Precision data for Levofloxacin

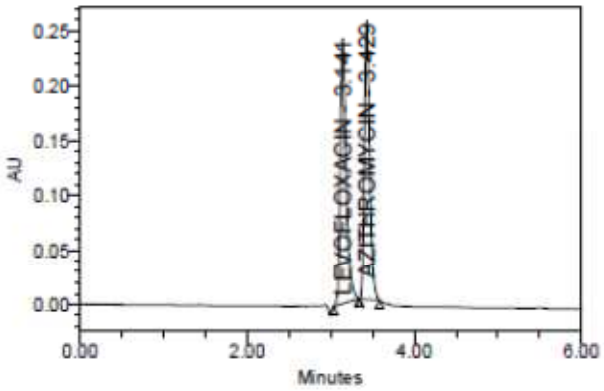
S.no	RT	Area	%Assay
injection1	3.144	1130289	98
injection2	3.141	1135307	99
injection3	3.142	1136018	99
injection4	3.145	1131067	99
injection5	3.142	1130362	99
injection6	3.138	1133569	99
Mean			99
Std. Dev.			0.22
% RSD			0.22

Table 18: Precision data for Azithromycin

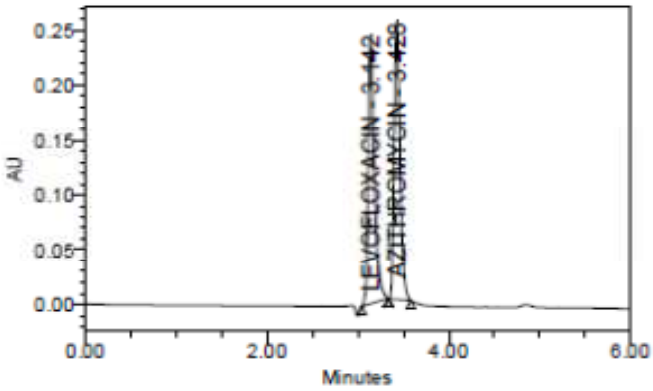
S.no	RT	Area	%Assay
injection1	3.431	1112555	100
injection 2	3.429	1116367	100
injection 3	3.428	1113656	100
injection 4	3.432	1113585	100
injection 5	3.430	1114698	100
injection 6	3.428	1114883	100
Mean			100
Std. Dev.			0.12
%RSD			0.12



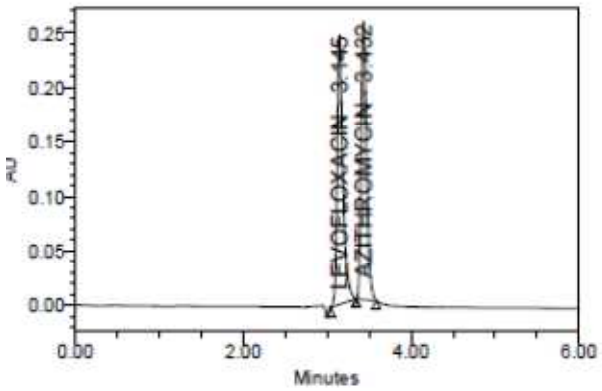
Chromatogra m for precision injection 1



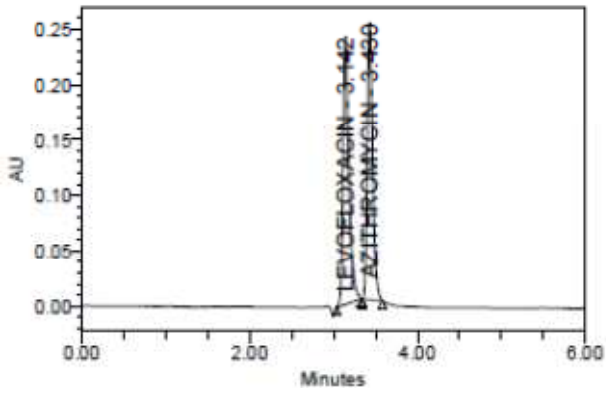
Chromatogram for precision injection 2



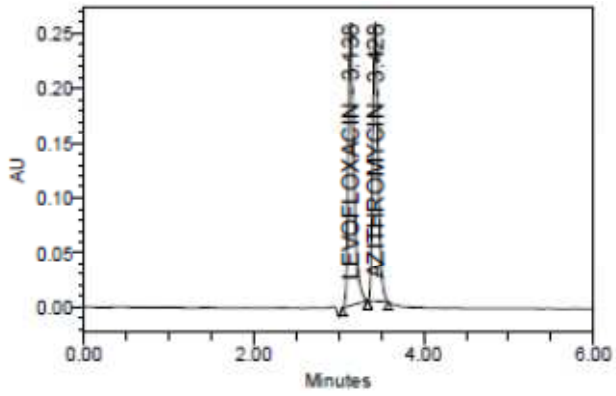
Chromatogram for precision injection 3



Chromatogram for precision injection 4



Chromatogram for precision injection 5



Chromatogram for precision injection 6

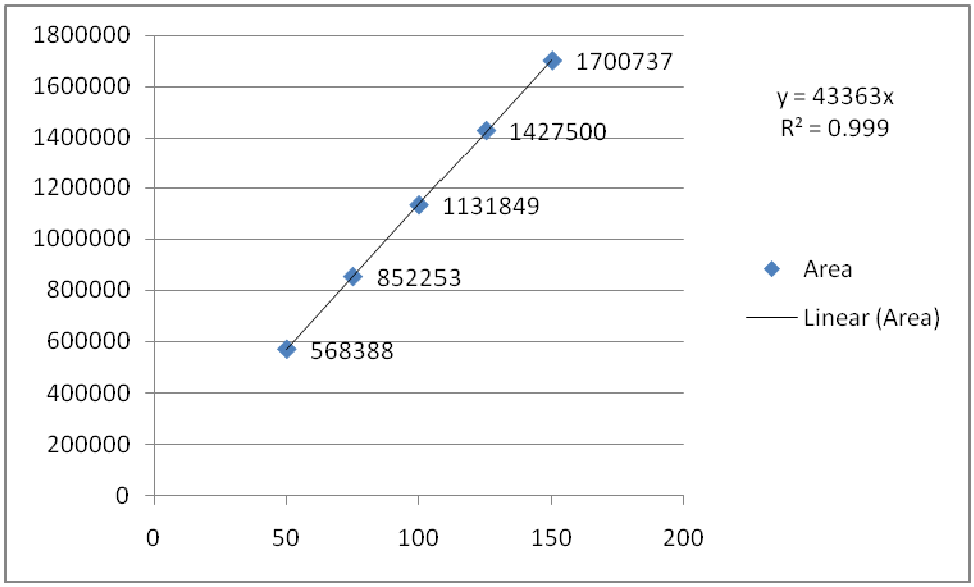
RESULT

Results of variability were summarized in the above table. % RSD of peak areas was calculated for various run. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise**.**

LINEARITY:

Table 19: Linearity data for Levofloxacin

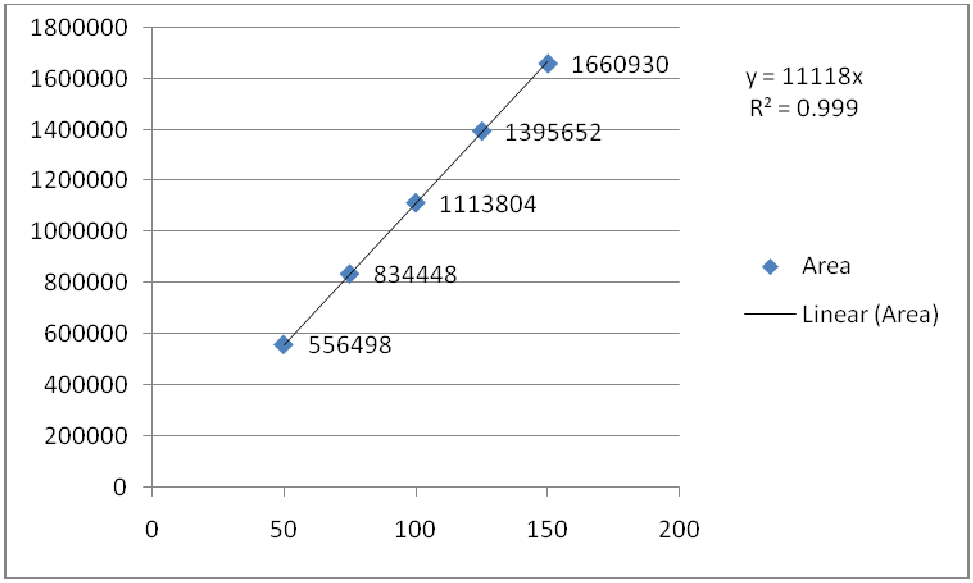
s.no	Conc(µg/ml)	RT	Area
1.	50	3.155	568388
2.	75	3.146	852253
3.	100	3.136	1131849
4.	125	3.139	1427500
5.	150	3.137	1700737
Std.dev			
Slope			
Intercept			
Correlation coefficient (r^2)			0.999



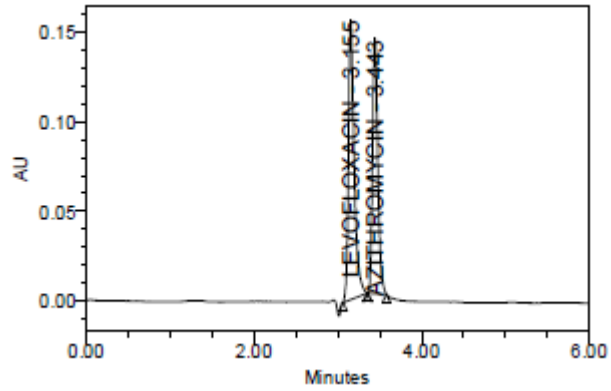
Linearity plot of Levofloxacin

Table 20 : Linearity data for Azithromycin

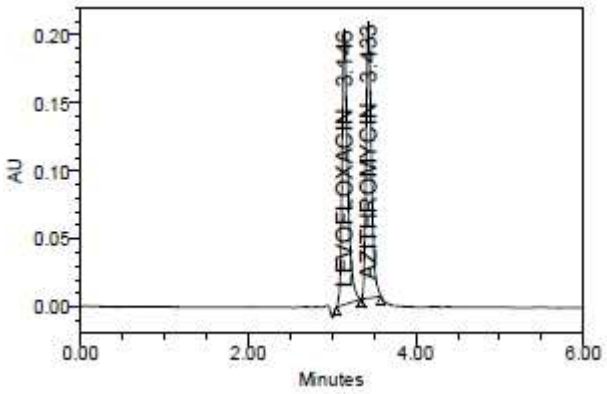
s.no	Conc(µg/ml)	RT	Area
1.	50	3.443	556498
2.	75	3.433	834448
3.	100	3.426	1113804
4.	125	3.431	1395652
5.	150	3.428	1660930
Std.dev			
Slope			
Intercept			
Correlation coefficient (r^2)			0.999



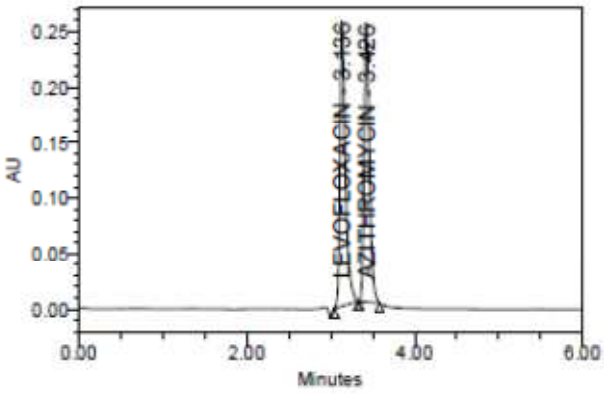
Linearity plot of Azithromycin



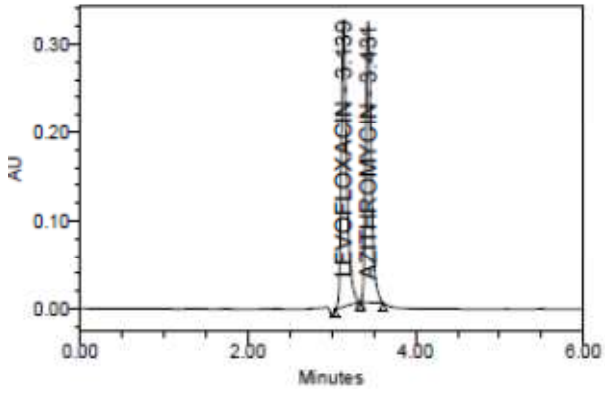
Chromatogram representing lineartity 1



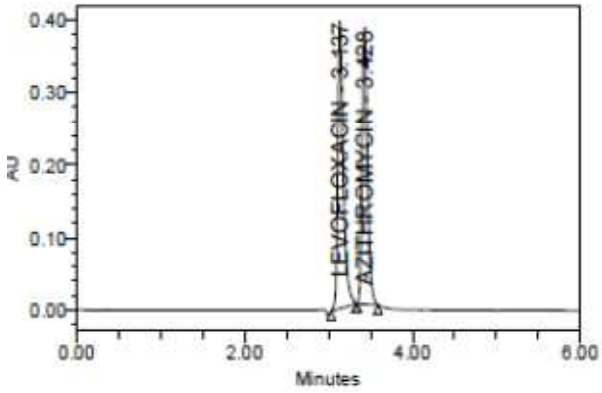
Chromatogram representing linearity 2



Chromatogram representing linearity 3



Chromatogram representing linearity 4



Chromatogram representing linearity 5

RESULT

A linear relationship between peak areas versus concentrations was observed for Levofloxacin and Azithromycin in the range of 50% to 150% of nominal concentration. Correlation coefficient was 0.999 for both Levofloxacin and Azithromycin which prove that the method is linear in the range of 50% to 150%.

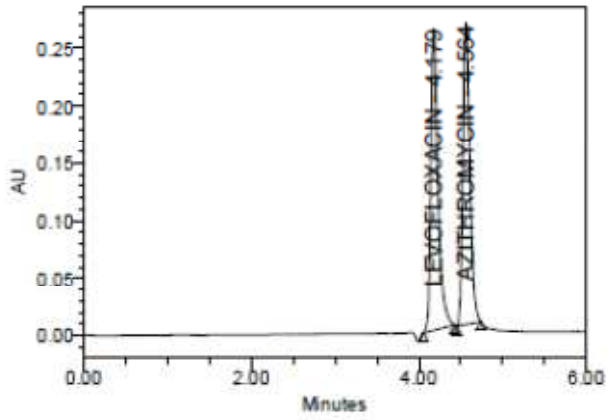
6. ROBUSTNESS:

Table 21 : Robustness data for Levofloxacin

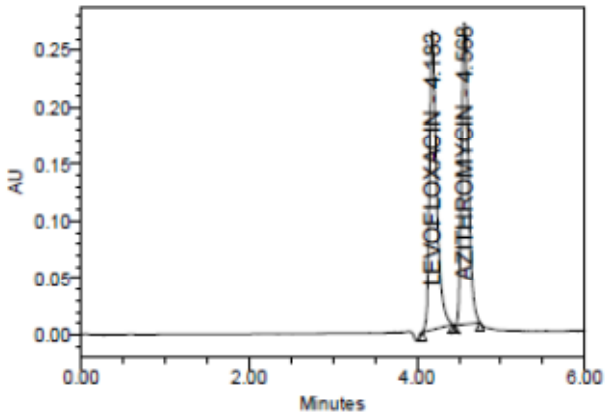
parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate(0.8ml/min)	4.178	12562	1.52
Actual flow ate(1.0ml/min)	3.154	11143	1.49
Increased flow rate(1.2ml/min)	2.516	10342	1.40
Decreased temperature(20^0c)	4.179	12660	1.52
Actual temperature(25^0c)	3.154	11143	1.49
Increased temperature(30^0c)	4.183	12750	1.52

Table 22: Robustness data for Azithromycin

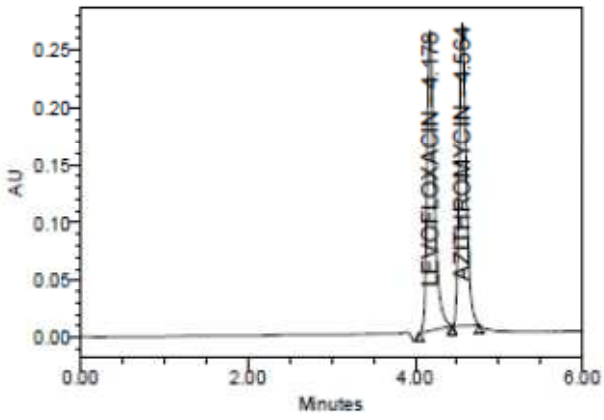
parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate (0.8ml/min)	4.564	14378	1.30
Actual flow rate (1.0ml/min)	3.439	13998	1.30
Increased flow rate (1.2ml/min)	2.746	12574	1.32
Decreased temperature(20^0c)	4.564	14785	1.28
Actual temperature(25^0c)	3.439	13998	1.30
Increasedtemperature(30^0c)	4.568	14978	1.30



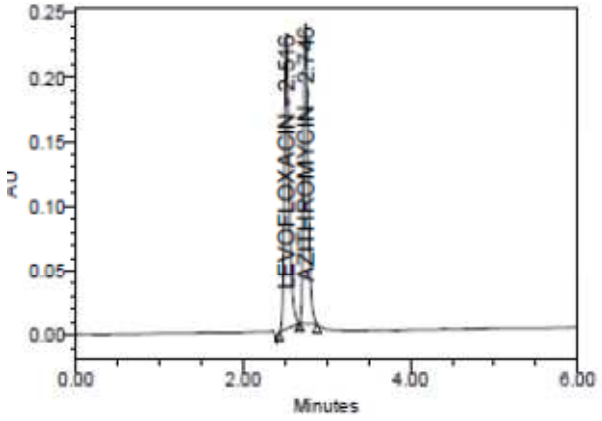
Chromatogram for decreased flowrate



Chromatogram for increased flowrate



Chromatogram for decreased temperature



Chromatogram for increased temperature

RESULT

The results of Robustness of the present method had shown that changes made in the Flow and Temperature did not produce significant changes in analytical results which were presented in the above table. As the changes are not significant we can say that the method is Robust.

7. LIMIT OF DETCTION:

Minimum concentration of standard component in which the peak of the standard gets merged with noise called the LOD

$$LOD = 3.3* \sigma/S$$

Where;

σ = standard deviation

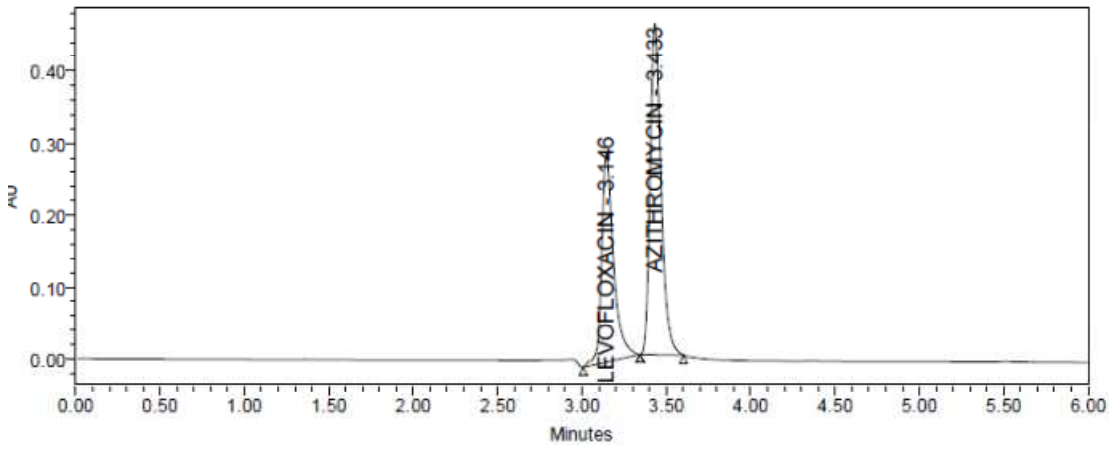
S = slope

LOD for Levofloxacin = 2.830

LOD for Azithromycin =2.9412

LOD data for Levofloxacin and Azithromycin

s.no	Sample name	RT	Area
1	Levofloxacin	3.146	1411711
2	Azithromycin	3.433	2061485



Chromatrogram for LOD

8. LIMIT OF QUANTIFICATION:

Minimum concentration of standard component in which the peak of the standard gets detected and quantification

$$LOQ = 10*\sigma/S$$

Where;

σ = standard deviation

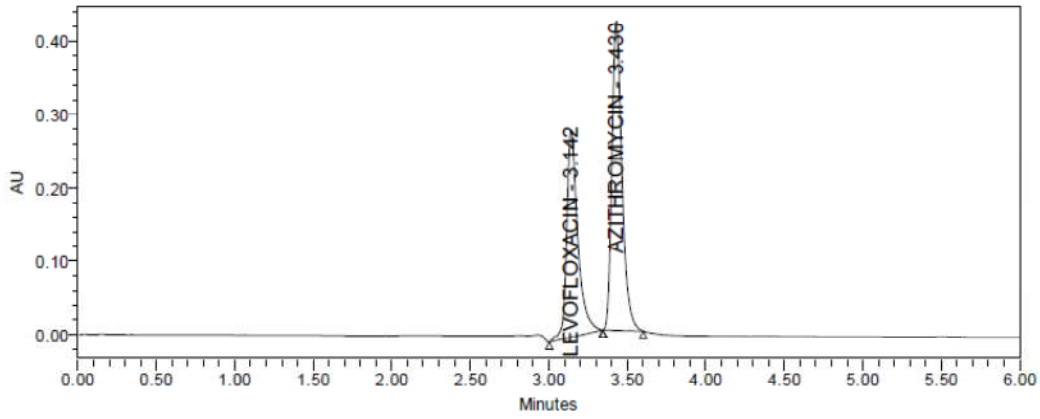
S = slope

LOQ for Levofloxacin =9.434

LOQ for Azithromycin =9.8039

LOQ data for Levofloxacin and Azithromycin

S.no	Sample name	RT	Area
1	Levofloxacin	3.142	1380760
2	Azithromycin	3.430	1866930



Chromatogram for LOQ

ASSAY:

Table 23 : Assay data for Levofloxacin and Azithromycin

	Levofloxacin		**Azithromycin**	
S.No	**Standard Area**	**Sample area**	**Standard Area**	**Sample area**
1	1130289	1131289	1112555	1111555
2	1135307	1133307	1116367	1112367
3	1136018	1134018	1113656	1110656
4	1131067	1131067	1113585	1113585
5	1130362	1129362	1114698	1114698
Average	113268.6	113172.4	111417.2	1112572
Tablet average weight	1383.0		1383.0	
Standard weight	500		500	
Sample weight	1383.0		1383.0	
Label amount	500		500	
std.purity	99.1		99.7	
%Assay	99.31		99.40	

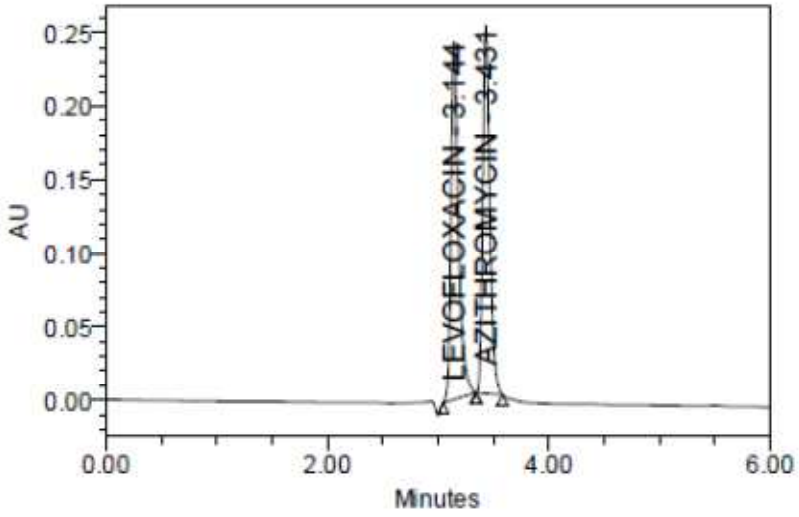


Fig. no. 46: Chromatogram for assay of sample 1

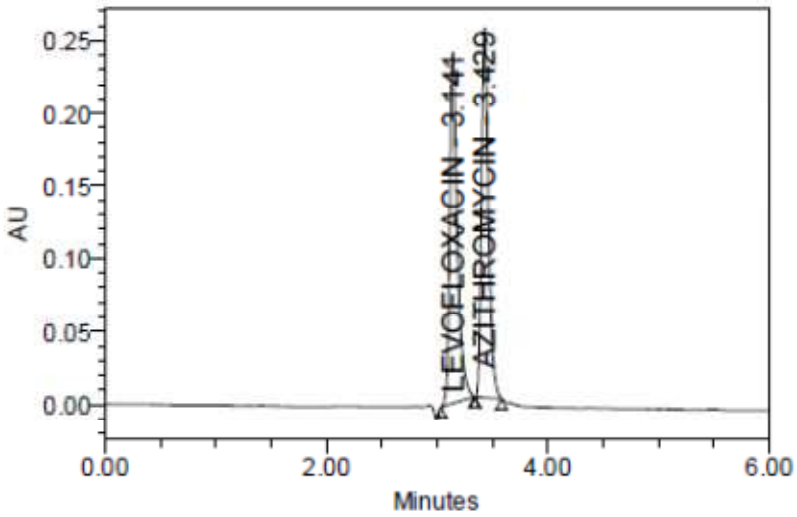


Fig. no.47: Chromatogram for assay of standard 1

Result:

The % assay of the Levofloxacinwas found to be 99.31% and for Azithromycinis 99.40%.

8. CONCLUSION

The study is focused to develop and validate HPLC methods for estimation of Levofloxacin and Azithromycin in tablet dosage form.

For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation steps. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool.

The method shows good reproducibility and good recovery. From the specificity studies, it was found that the developed methods were specific for Levofloxacin and Azithromycin

SUMMARY

SUMMARY OF LEVOFLOXACIN :

Summary of validation data for levofloxacin

S.NO	PARAMETER	RESULT	ACCEPTENCE CRITERIA
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 14206 1.35 3.154 0.3	 Not less than 2500 Not more than2 Not more than 2%
2	Specificity a) Blank interference b) Placebo interference	 Specific	 Specific
3	Method precision(%RSD)	0.22	Not more than 2.0%
4	Linearity parameter Slope Intercept Correlation coefficient(r^2)	50-150 mcg/ml 0.999	 Not less than 0.999
5	Accuracy (Mean % recovery) 50% 100% 150%	 100% 100% 100%	 97.00 – 103.00%
6	Robustness a) Flow rate variation b) Temperature variation	All the system suitability parameters are within the limits.	

SUMMARY OF AZITHROMYCIN :

Summary of validation data for Azithromycin

S.NO	PAAMETER	RESULT	ACCEPTENCE CRITERIA
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 11075 1.55 3.436 0.2	 Not less than 2000 Not more than 2 Not more than 2
2	Specificity c) Blank interference d) Placebo interference	 Specific	 Specific
3	Method precision(%RSD)	0.12	Not more than 2.0%
4	Linearity parameter Slope Intercept Correlation coefficient(r^2)	50-150 mcg/ml 0.999	 Not less than 0.999
5	Accuracy (Mean % recovery) 50% 100% 150%	 100 100 100	 97 - 103%
6	Robustness c) Flow rate variation d) Temperature variation	All the system suitability parameters are within the limits.	

BIBLIOGRAPHY

1. Sharma BK. Instrumental methods of chemical analysis, Introduction to Analytical chemistry, 23thed.Goel Publishing House Meerut, 2004, P12-23.
2. H.H. Willard, L.L. Merritt, J.A. Dean, F.A. Settle. Instrumental Methods of Analysis, 7th edition, CBS publishers and Distributors, New Delhi. 1986, P.518-521, 580-610.
3. John Adamovies. Chromatographic Analysis of Pharmaceutical, Marcel Dekker Inc. New York, II Ed, P.74, 5-15.
4. GurdeepChatwal, Sahm K. Anand. Instrumental methods of Chemical Analysis, 5th edition, Himalaya publishing house, New Delhi, 2002, P.1.1-1.8, 2.566-2.570
5. D. A. Skoog. J. Holler, T.A. Nieman. Principle of Instrumental Analysis, 5th edition, Saunders College Publishing, 1998, P.778-787.
6. Skoog, Holler, Nieman. Principals of Instrumental Analysis,5thEdition, HarcourtPublishers International Company, 2001, P.543-554.
7. William Kemp. Organic Spectroscopy, Palgrave, New York,2005,P.7-10, 328-330
8. P.D. Sethi. HPLC: Quantitative Analysis Pharmaceutical Formulations, CBS Publishers and distributors, New Delhi (India), 2001, P.3-137.
9. Michael E, Schartz IS, Krull. Analytical method development and Validation. 2004. P. 25-46.
10. R. Snyder, J. Kirkland, L. Glajch. Practical HPLC method development, II Ed, A Wiley International publication, 1997, P.235,266-268,351-353.653-600.686-695.

11. Basic Education in Analytical Chemistry. Analytical Science. 2001:17(1).
12. Method validation guidelines International Conference on harmonization; GENEVA; 1996
13. Berry RI, Nash AR. Pharmaceutical Process Validation, Analytical method validation, Marcel Dekker Inc. New work. 1993; 57:411-28
14. Anthony C Moffat, M David Osselton, Brian Widdop. Clarke's Analysis of Drugs and Poisons, Pharmaceutical Press, London, 2004, PP 1109-1110, 1601-1602.
15. Klaus Florey, Analysis Profile of Drugs Substances, Academic Press, New York, 2005, P.406-435.
16. P.N. Arora, P.K. Malhan. Biostatistics, Himalaya Publishers House, India, P.113,139-140,154.
17. Doserge, Wilson and Gisvold's text book of organic medicinal and pharmaceutical chemistry, 8th edn, Lippincott Company, 1982, P.183-197.
18. http//en.wikipedia.org/wiki/Chromatography.
19. Azithromycin Drug profile: www.drugbank.ca/drugs/**DB00207** .
20. Levofloxacin Drug profile.www.drugbank.ca/drugs/**DB01137**.
21. EnjemKarunaker Reddy*etal.*;Development And Validation Of RP-HPLC Method For TheSimultaneousEstimation Of Azithromycin And Levofloxacin In Combined Tablet Dosage.FORM International Journal of Pharmaceutical Sciences and Research 2013.
22. **Yun Nairu1***et al.*;RP-HPLC method for determination of levofloxacin and azithromycin concentration at equal pace in medium of CM129.
23. M. Rachidi *et al.*;New Spectrophotometric Method for Azithromycin Determination

24. S.C. Arora*et al.*; Development, Characterization And Solubility Study Of Solid Dispersions Of Azithromycin Dihydrate By Solvent Evaporation Method.
25. MakarandAvhad*et al.*;Development And Validation Of Simultaneous UvspectrophotometricMethod For The DeterminationOf Levofloxacin And Ambroxol In Tablets
26. S. Schulte*et al.*;Determination of the Newer Quinolones Levofloxacin and Moxifloxacin in Plasma by High-Performance Liquid Chromatography with Fluorescence Detection.Journal of Chromatographic Science, Vol. 44, April 2006
27. Vishal Shah *et al.*;Development And Validation Of Derivative Spectroscopicmethod For Simultaneous Estimation Of CefiximeTrihydrateand Azithromycin Dihydrate In Combined Dosage Form.IJPSR, 2012; Vol. 3(6): 1753-1760